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On Protein Data Bank entries, bovine beta-trypsin, and the mechanism of action of calcium in trypsin autolysis

Buono, Ronald Alexander, Ph.D.

City University of New York, 1991

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On Protein Data Bank Entries, Bovine beta-Trypsin, and the Mechanism of Action of Calcium in Trypsin Autolysis

A

by

Ronald Alexander Buono

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1991

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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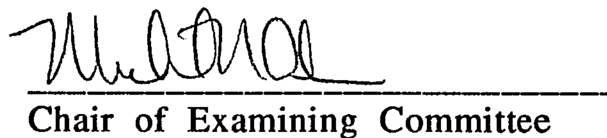
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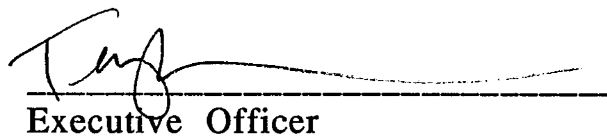
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Abstract

ON PROTEIN DATA BANK ENTRIES, BOVINE beta-TRYPSIN, AND THE MECHANISM OF ACTION OF CALCIUM IN TRYPSIN AUTOLYSIS

by

Ronald Alexander Buono

Advisor: Dr. Michael N. Liebman

A statistical analysis of comparisons among several types of crystalline proteins was conducted to construct a library of conformational changes in proteins with identical primary sequences. The resolution of these structures was not correlated with either their precision or their conformational variability. However, both conformational variability and the magnitude of reported thermal parameters were found to be correlated with distance from the center of mass of the proteins.

Specific structural and thermal parameter analyses indicated that bovine β-trypsin is a typical globular protein, with characteristics similar to most of the other structures deposited in the Protein Data Bank. The five uncomplexed trypsin structures were found to be highly homologous even though they had been crystallized under a variety of conditions. Furthermore, these analyses showed that the active site is particularly well-conserved, with the calcium-binding and autolysis sites exhibiting progressively greater conformational and thermal variabilities.

Molecular modeling of the β-trypsin autolysis dimer, using representative crystal structures and based on an analogy to the trypsin/pancreatic trypsin inhibitor (PTI) complex, showed that the P1'-P5' residues of the 'substrate' are responsible for the majority of the steric overlap in the dimer. Molecular mechanics minimizations indicated that backbone atoms must adapt significantly at the protein-protein interface in order to produce the Michaelis complex. The extent of this adaptation indicates that these changes are the result of an activated process between the 'enzyme' and 'substrate'.

Fourier-transform infrared spectroscopic experiments showed that no detectable conformational changes occur to the secondary structure of two inhibited forms of bovine β-trypsin upon calcium binding. Furthermore, the appearance of a band at 1699 cm^{-1} in benzamidine-inhibited trypsin is consistent with the observed asymmetric crystal structure interaction between the amidinium group of the inhibitor and the carbonyl group of 189D in the enzyme active site.

Modeling of the 16 basic amino acid sites of β-trypsin as potential substrates for autolysis, based again on the trypsin/PTI analogy, explained the specificity for the 145K site in the processing of β- to α-trypsin. Analyses of the solvent accessibilities, thermal parameters, and steric overlap resulting from dimerization at the 16 sites led to the conclusion that the 145K site is the preferred initial substrate in β-trypsin autolysis if mobility of the P1'-P5' residues is important for allowing bulk water to enter the active site for the deacylation step of proteolysis. A conformational change after cleavage of β-trypsin is also inferred from these analyses.

It has been proposed by others that calcium slows β-trypsin autolysis by the induction of a "conformational change" in the autolysis loop of the 'substrate'. However, the combination of molecular modeling, crystal structure and thermal parameter analyses, FTIR spectroscopic evidence, and consideration of other experiments performed on both trypsin and chymotrypsin, suggest that calcium's mechanism of action is of a specific electrostatic origin.

Preface

Improvements in both experimental and computational technologies have accelerated the amount of scientific work done on protein structure and function and will continue to do so for the foreseeable future. The increase in the number of protein and polypeptide structures solved by X-ray crystallography is the single most important experimental impetus behind current computational work. Indeed, theoreticians owe much of their scientific existence to the crystallographers' work.

At issue is not whether experimentalists and theoreticians will continue to advance independently of each other, but to what extent their work will increasingly validate, enhance, and motivate each others. Certainly, today, the burden of scientific justification falls on the theoreticians. No computational work, no matter how thoroughly and precisely done, can truly be regarded as scientific achievement without experimental verification, corroboration, or predictive ability. But as the number of computational achievements which attain these requirements increases, so too does their validity and the confidence in them to 'stand-alone'. Future theoretical work will, most probably, be predictive and indicate pertinent and efficient experimentation to be done in the fields of pharmacology, biophysics, and genetic engineering.

No phenomena tests this tenant better than the association of two proteins and no system serves as a better model than β-trypsin autolysis. Protein-protein associations are an integral part of all life processes. Most, by nature, are highly specific, but short-lived, and thus are not amenable to crystallographic investigation. Trypsin associates with most proteins, including other trypsins, and hydrolyzes them. It is physiologically important and complex; is ubiquitous and readily available; is relatively stable; has been the subject of thousands of experimental studies; has been crystallized in numerous forms; and is small enough to study by some contemporary computational methods. It is, thus, an ideal system to test the relationship between theory and experiment.

Acknowledgements

Thanks and all my love to my family and friends
who helped and supported me through these times.
Especially my mother, Joan Marie Nash Buono,
Susanne and, just recently, Eric.

Dedication

Dedicated to the memories of
my father, Philip Lawrence Buono,
my father-in-law, John Joseph Stigle,
and my dear friend, David Gunning Hine.
We miss you.

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Background

Trypsin (EC 3.4.21.4) is a mammalian protease which is is also found in fish, insects, plants, and bacteria (Stroud et al., 1974). In mammals, it is synthesized and secreted by the beta cells of the pancreas as its inactive zymogen. Historically, it was the first protein to which the word "enzym" was applied to - almost 115 years ago (Kuhne, 1877). The zymogen consists of 229 amino acids while the native enzyme results from the removal of six residues from the N-terminus by the action of membrane-bound enterokinase or by trypsin itself. The molecular weight of the native enzyme is approximately 23,500. Its pH optimum for catalytic activity is 8 and its pI is 10.5 due to a composition of 16 basic amino acids and 10 acidic amino acids. It's zymogen has been postulated to be as old as the divergence of eukaryotic organisms (Olson, 1970) - about four hundred million years (Dickerson, 1971).

Trypsin, along with two other pancreatic enzymes, chymotrypsin and elastase, is a serine protease. Serine proteases are enzymes characterized by the presence of the Asp-His-Ser "catalytic triad" (Weiner et al., 1986; Warshel & Russel, 1986). Trypsins hydrolyze peptides and esters at bonds involving the carbonyl groups of arginine or lysine. Chymotrypsins have specificity for aromatic and hydrophobic amino acids. Elastase has specificity principally for elastin, but also for uncharged, non-aromatic amino acids. The differing specificity of these serine proteases is conferred by sidechains of amino acids which comprise the remainder of the active site. The specificity of trypsin for basic amino acids is conferred by the acidic amino acid, aspartic acid 189, which is situated at the bottom of the active site (Graf et al., 1988).

Pancreatic juice, consisting of digestive enzymes, water, and various electrolytes, is drained from the main pancreatic duct of Wirsung and the accessory pancreatic duct of Santorini into the duodenum at the Sphincter of Oddi, approximately 8 cm from the pylorus. Here, in conjunction with bile secreted by the liver, trypsin and other digestive

enzymes catabolize food which has been acted on previously by salivary enzymes, gastric acid, and pepsin secreted by the gastric mucosa.

Calcium plays a central role in the regulation of a multitude of enzymes synthesized and secreted by the pancreas. Pancreatic exocrine secretion is isotonic with plasma, with calcium concentrations being 2-8 micromole/ml juice in the guinea pig, depending on experimental conditions (Ceccarelli et al., 1975). Calcium is secreted by two mechanisms, one associated with secretory proteins and the other directly from the extracellular fluid by traversing the paracellular pathways (Jansen et al., 1980). Calcium's regulatory role toward various proteins occurs at four levels: secretion rate (Williams & Hootman, 1986); synthesis rate (Stratowa & Rutter, 1986); maintenance of conformational stability and enzymatic activity (Drakenberg et al., 1984; Howat & Sarles, 1979); and prevention of proteolysis (Poulos & Price, 1972).

Trypsinogen binds two calciums. One binds weakly at the N-terminal six residues with a pKa $\simeq$ 1.8 (Delaage & Lazdunski, 1967). Four of these residues are acidic amino acids. This hexapeptide is hydrolyzed during zymogen activation. Both the native enzyme and the zymogen possess a high affinity calcium binding site, with a pKa $\simeq$ 3.4 (Abita et al., 1969). This site is located in a loop of 13 amino acids, identified as residues 68 to 80 in the chymotrypsinogen numbering sequence (Bode & Schwager, 1975a).

The weakly bound calcium accelerates the conversion of trypsinogen to native trypsin by both enterokinase (Baratti et al., 1973) and trypsin (McDonald & Kunitz, 1941). Conversely, the tightly bound calcium slows both β- (Bier & Nord, 1951a, 1951b ; Gorini, 1951; Green et al., 1952) and α-trypsin (Abbott et al., 1975) autolysis.

The identity of the calcium binding site and the effects of calcium binding on the trypsin structure have been the subjects of intense experimental investigation. Historically, the X-ray crystallographic identification of the binding site proved difficult and was not unequivocally shown until 1975. The structure of benzamidine-inhibited bovine β-trypsin (BA-trypsin) was found to have a single site which met all of the available criteria for a

calcium ion during the course of constrained crystallographic refinement (Bode & Schwager, 1975b). This site was identified as a loop formed by residues 68-80 and was found to contain two glutamic acid residues previously assigned as glutamines.

Calcium has been found to stabilize trypsin and [apparently] increase its catalytic activity (Kunitz, 1934; Bier & Nord, 1951a, 1951b; Gorini, 1951). Along with calcium, the divalent cations manganese and cadmium (Nord & Bier, 1953), and cobalt and cadmium (Green et al., 1952), have also been found to stabilize trypsin. The increase in catalytic activity upon calcium binding is not thought, however, to result from a 'superactivated' structure with increased intrinsic activity (Sipos & Merkel, 1970). Rather it is thought that the increased activity is a result of an increase in the concentration of the active enzyme because of protection from autolysis (Liebman, 1986).

The stabilizing effect of calcium on trypsin has been confirmed subsequently by Lazdunski and Delaage (1965), Sipos and Merkel (1970), and Gabel and Kasche (1973). All three of these groups concluded that trypsin takes on a "more compact" structure upon calcium binding as indicated by optical rotary measurements, ultraviolet differential spectroscopy, and fluorescence measurements respectively.

For more than 30 years, the utilization of fluorescence emissions of proteins has been beneficial in showing that it is affected by secondary and tertiary structure, along with higher levels of organization in proteins (Udenfriend, 1962). Tyrosine and tryptophan fluorescence and phosphorescence emission are sensitive to environment, with changes to a more hydrophobic environment leading to blue shifts (Arrio et al., 1973). Because light emission takes place on a much longer time scale than light absorption (10^{-9} to 10^{-8} vs. 10^{-15} sec), a wide range of interactions and perturbations can influence the emission spectrum. Among these perturbations are protonation and deprotonation, local conformational changes and changes to hydrogen bonding patterns, solvent-cage relaxation, and processes coupled to translational and rotational motions. The three aromatic amino acids, tryptophan, tyrosine, and phenylalanine absorb at 280, 274, and 257 nm and emit at 348,

303, and 282 nm respectively. Another beneficial feature of fluorescence is the ability of other chromophores, separated by distances up to 80 Å, to cause quenching by a dipole-dipole mechanism (Cantor & Schimmel, 1980).

Direct investigation of calcium binding to molecules of biological interest is difficult for two reasons. First, the electronic transitions of calcium cannot be studied by conventional optical absorption and emission spectroscopy. Second, the lack of unpaired electrons precludes the use of nuclear magnetic resonance or electron spin resonance methods to probe the chemical or structural nature of calcium binding sites.

Enhanced terbium luminescence has become a tool for probing calcium binding sites because terbium, a lanthanide metal, contains an unpaired f electron. This makes it amenable to study with magnetic resonance techniques. Both calcium and terbium also have similar ionic radii and both display variable coordination numbers and lack pronounced directionality in binding to donor groups.

Excitation of lanthanide f-f emitting states is required for their use as probes. This excitation may be either direct or indirect. Because of the weak absorptivities of lanthanide transitions in the visible and near uv spectral region, direct excitation requires either very high concentrations ($>10^{-2}$ M) or a powerful excitation source, such as a laser. Horrocks et al. (1977) used a pulsed laser source to estimate the number of water molecules bound to lanthanides in a variety of complexes, including that with thermolysin. The number of bound water molecules was estimated from lifetime and emission decay characteristics as function of the D_2O content of the solvent water.

Indirect excitation of lanthanides involves excitation of ligand chromophores, such as highly molar absorptive aromatic amino acids, by near uv radiation (Brittain, 1976). Non-radiative energy transfer from the sidechain to terbium can occur by a Forster dipole-dipole resonance energy transfer mechanism. This energy transfer is almost independent of angle and depends only on the distance between the donor and acceptor as r^{-6} (Fortser,

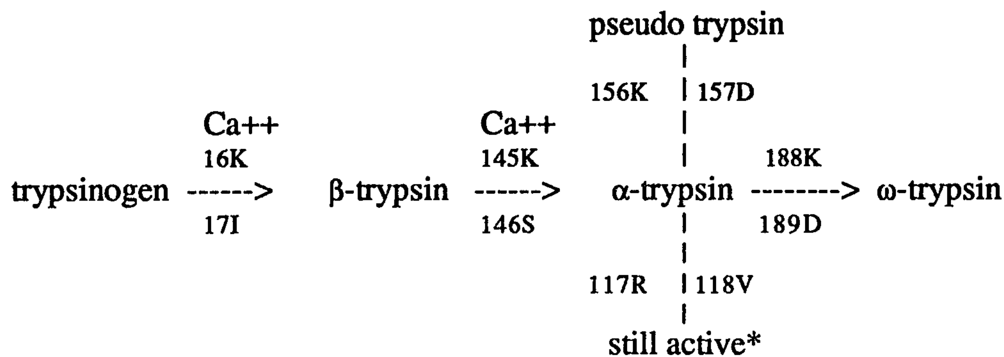
1959). This energy transfer can lead to greatly enhanced (up to 10^5 fold) terbium phosphorescence on the green spectral region (535 - 555 nm).

More specific calcium effects have been found by difference ultraviolet spectra (Sipos & Merkel, 1970; Matsushima et al., 1971, Gabel & Kasche, 1973; DeJersey et al., 1980) and the enhancement of the green luminescence of terbium ion and "energy transfer" involving a tryptophan residue and the fluorescent lanthanide terbium (DeJersey et al., 1980; Epstein et al., 1974, 1977). All of these experimentalists attributed their results to the presence of 141W, an amino acid about 10 Å from the bound calcium. However, Arrio et al. (1973) found no spectroscopic evidence that the intrachain splits in the three physiological forms of trypsin have an effect on any tryptophan residues. Binding of competitive inhibitors to the active site showed, however, that at least one tryptophan residue is involved in conformational changes upon binding to the active site.

Several enzymes, other than trypsin, are influenced in their activity and physical properties by calcium binding. Proteinase K undergoes triggered "concerted domino-like movement of five peripheral loops and two alpha helices" with calcium removal (Bajorath et al., 1989). The geometry of the secondary binding site and the active site (25 Å from the calcium) is also affected and the enzyme cannot fully recover its activity after calcium is added back.

Deoxyribonuclease exhibits both CD and ORD changes upon calcium binding and requires calcium to hydrolyze DNA (Poulos & Price, 1972). The authors propose that calcium binding induces a conformational change "leading to a more compact structure." It is further connected by the fact that calcium protects it against inactivation by both trypsin and chymotrypsin (Poulos & Price, 1972). The systems are different from trypsin autolysis, however, in that manganese does not protect it completely. Thermomycolase is also protected by calcium against autolysis and thermal urea degradation (Voorduow & Roche, 1975). Finally, ovomucoid antitrypsin does not aggregate in the presence of calcium (Bier & Nord, 1953).

Below is a schematic diagram of trypsin processing:



* Maroux et al. (1967) have shown that the 117R cleaved form retains partial activity.

Endogenous inhibitors of the serine proteases are stored in zymogen granules and are also secreted in the pancreatic juice. In man, the inhibitor of trypsin is termed pancreatic secretory trypsin inhibitor (STI) and belongs to the Kazal family of protease inhibitors (Laskowski, Jr. & Kato, 1980). Its function is to prevent unwanted proteolysis and zymogen activation. Other classes of proteinase inhibitors exist, with the most well known and studied being pancreatic trypsin inhibitor (PTI), a polypeptide of 58 amino acids which is found only in bovine and caprid. This was the first inhibitor to be isolated in crystalline form (Huber et al., 1970), sequenced (Kassell & Laskowski, 1965), and structurally determined by X-ray crystallography (Deisenhofer & Steigemann, 1975). It has also been the object of numerous theoretical studies involving protein folding (Levitt, 1976), nmr analysis (Wagner et al., 1987), and molecular dynamics calculations (Karplus & McCammon, 1981).

Every endogenous serine protease inhibitor contains a surface peptide bond at the P1 residue (Schechter & Berger, 1967), called the reactive site, which associates to the active site of the protease in a substrate-like manner. Although their overall folding is different, the geometry of the reactive site regions are almost identical. They are encompassed by a disulfide bond and are in the conformation of an ideal substrate.

In the crystal complex the peptide bond of the P1 residue is intact but the carbonyl carbon is pyramidalized by its interaction with the 'oxyanion hole' of the enzyme

(Ruhlmann et al., 1973; Huber et al., 1975). The oxyanion hole is comprised of the NH groups of both 193G and 195S. While k_{cat}/K_m is large for this association, both k_{cat} and K_m are very small. Inhibitor hydrolysis is extremely slow due to the exclusion of solvent water, the nucleophile required for the deacylation step (Ruhlmann et al., 1973; Blow et al., 1972). More recently it has been hypothesized (Dufton, 1990) that these inhibitors may act by not inducing domain movement necessary for stressing the reactive site scissile peptide bond. The system thus behaves as a simple equilibrium process between the free enzyme and inhibitor and the complex. Association constants for the interaction are typically on the order of 10^9 - 10^{18} (Laskowski, Jr. & Kato, 1980).

The dramatic increase in computational and experimental methods involving protein structure determination and analyses over the past twenty years has led to a plethora of scientific studies on them. X-ray crystallography, fluorescence spectroscopy, and more recently, nmr and Fourier- transform infrared spectroscopy, have proved to be valuable in understanding protein structure and its relation to function.

Crystal structure determinations of amino acids and polypeptides (Marsh & Donohoe, 1967) preceded the determination of protein structures. About 400 crystal coordinates of proteins are currently deposited in the Protein Data Bank (Bernstein et al., 1977), a national resource devoted to the dissemination of these data. The crystal conformations of polypeptides (Lautz et al., 1990) and proteins (Wagner et al., 1987) have also been shown to be similar to that in solution.

Miyazawa and Blout (1961) pioneered the use of infrared spectroscopy as a tool for deducing protein structure by concentrating on the Amide I region (Amide I' in deuterated proteins). Absorbance in this region results primarily from stretching vibrations of the C=O backbone groups. It is thus sensitive to the molecular geometry and hydrogen bonding patterns of the protein backbone and is indicative of secondary structure elements such as alpha helices, beta sheets, and turns (Byler & Susi, 1986).

More recently, resolution enhancement (Susi & Byler, 1983; Byler & Susi, 1986) and curve-fitting techniques have been used to correlate specific folding types and individual component bands in the infrared spectrum (Byler & Susi,1986; Lee et al., 1986; Susi & Byler, 1986; Surewicz & Mantsch, 1988). Correlation of spectra of β-, α-, and diisopropylfluorophosphate-inhibited trypsins with high-resolution X-ray structures by Prestrelski et al. (1991) enabled the assignment of a component of the absorption at 1655 cm^{-1} to the autolysis loop of β-trypsin. Absorption at this frequency had previously been assigned only to alpha helical structure (Byler & Susi, 1986; Surewicz & Mantsch, 1988).

This assignment makes absorption at 1655 cm^{-1} a useful tool for studying calcium's effect on trypsin autolysis. Previously alluded to experimental work showed that calcium changes some physical properties of trypsin upon its binding. This, in turn, led many investigators (see Chapter 6) to hypothesize that the mechanism of action of calcium in slowing β-trypsin autolysis is alteration of its autolysis loop conformation of into one which is unable to bind to the active site of another β-trypsin acting as the 'enzyme'.

Computational analyses of proteins has taken on many forms, including, but not limited to: molecular orbital studies (Weiner & Kollman, 1986; Warshel & Russell, 1986; Kubodera, et al., 1990); molecular mechanics calculations (McCammon et al., 1976); molecular dynamics simulations (McCammon & Karplus, 1980); quantitization of intermolecular interactions (Blaney et al., 1982); B factor relationships (Artymiuk et al., 1979); and the determination of amino acid side chain contacts (Janin et al., 1978) and orientations in proteins (Bhat et al., 1979).

B values, or Debye-Waller Factors, are a measure of the probability of finding an atom at a given distance from its equilibrium position in a crystal. This experimental parameter is derived from X-ray crystallography diffraction intensities by assuming that each atom is in harmonic motion in a quadratic potential well. In the refinement procedure individual atomic contributions to structure factors are multiplied by a factor $e^{-B\sin2q/\lambda2}$, where q is the Bragg angle of reflection and λ the wavelength of the X-rays. Mean-square

displacements ($<x^2>$) are related to B values by the relation $B = 8\pi^2<x^2>$ and are considered to have considerable relative accuracy within protein structures (Petsko & Ringe, 1984).

The electron density derived from X-ray crystallography measurements reflects the average position of that atom in every unit cell of the crystal. There are three contributions to this average position: vibrational motion; static disorder; and static lattice disorder. Static disorder results from molecules existing in multiple discrete states which are highly populated. Static lattice disorder results from imperfect crystals which lead to inhomogenous environments of the crystalline unit cells. Dynamic disorder results from the vibrational and static disorder contributions only, and have been shown to account for about 85% of $<x^2>$ in metmyoglobin (Hartman, 1982).

B values have also been shown to be correlated to solvent accessibility and distance from center of mass in lysozyme (Artymiuk et al., 1979; Sternberg et al., 1979) and metmyoglobin (Frauenfelder et al., 1979). Furthermore, a correlation has been found between charge on amino acid sidechains and their thermal variability. Frauenfelder et al. (1979) found that charged residues have more variability than comparably exposed neutral amino acids and described metmyoglobin as having a " . . . condensed core around the haem, semi-liquid regions towards the outside . . . ".

Comparison of the three dimensional structures of these molecules is a natural consequence of solving their crystal structures. Cullis et al. (1962) attempted the first comparison of protein structures. Their visual approximation of the similarity between horse oxyhemoglobin and whale myoglobin became supplanted by the root-mean-square computational techniques of Muirhead et al. (1967), Rao and Rossman (1973), and Liebman (1985a). Two, more recently applied, techniques have proved useful in protein crystal structure analyses. Distance matrix analysis was utilized by Liebman (1980) in studies on several different PDB structures. Difference linear distance plot analysis was utilized by Liebman et al. (1985a) in studies on carboxypeptidase A and by Liebman (1986)

on trypsin. Today, the wealth, accuracy, and reproducibility of the crystal structures of proteins enables investigators to not only deduce similarities between structures, but also subtle differences which are the consequence of chemical and environmental forces.

Three types of protein motion are considered to occur in proteins (Petsko & Ringe, 1984). Atomic fluctuations result from vibrations and range to 1 Å. Collective motions range to 5 Å and break down into fast and slow types. Triggered conformational changes range to 10 Å and result from a response to a specific stimulus, with the energy of the change coming from specific electrostatic and hydrogen bond interactions.

Molecular modeling of protein-protein associations, based on structures determined by X-ray crystallography, was pioneered by Blow et al. (1972). In that study, an attempt was made to model the association of bovine pancreatic trypsin with both chymotrypsin and trypsin, based on independent structure determinations. The enzymes and inhibitor were assumed to remain in their X-ray conformation and the surface sidechains were allowed "some" flexibility. In the best orientation achieved, seven hydrogen bonds and about 200 van der Waals contacts were made between the inhibitor and each enzyme.

Wodak and Janin (1978) developed an automated computer procedure for systematically generating all possible modes of association between proteins and studied bovine PTI and trypsin as a test case. In this procedure, each residue was replaced by an "interaction center" with steric and energetic properties. The criteria for the selection of stable structures was minimization of this potential energy function. By this method, nine interactions with binding properties similar to the native complex, but different orientations, were found.

Papamokos et al. (1982) modeled the complexation of the third domain of Japanese quail ovomucoid inhibitor (a Kazal-type inhibitor) with trypsin, chymotrypsin, and elastase. Their modeling with computer graphics was based on the trypsin-bpti complex. "Stereochemically satisfying models" were obtained and detailed interactions were outlined. The authors also noted that the conformation of the reactive site loop (P2-P2') of the quail

ovomucoid inhibitor was similar to that of three other serine protease inhibitors (basic pancreatic trypsin inhibitor, *Streptomyces* subtilisin inhibitor, and soybean trypsin inhibitor).

At least three other systems of protein-protein association have been modeled to date. The electron transfer complex of cyt c - cyt b5 was first modeled by Salemme (1976). Surface topographies of irregular polyhedra of the molecules were generated by connection of the most exposed atoms of surface amino acids. Further checks were performed with rigid space-filling models. A least-squares fitting process optimized the complementary charge and steric interactions between the invariant positive amino acids around the heme crevice in cyt c and the negative groups about the heme crevice in cyt b5. Two complementary fits , 180° apart about an axis normal to the "saddle surfaces", were initially found with the constraints that the two heme prosthetic groups are approximately coplanar and are no closer than 8.4 Å apart. Further checks led to the finding of prohibitive close contacts which necessitated rotation of the CB-CG bonds of lysine residues on cyt c for alleviation of the overlap.

Modeling of the association between cyt c and cyt b5 was later advanced with molecular dynamics calculations (Wendoloski et al., 1987). Their analysis found that electrostatic interactions at the interface resulted in a "flexible association complex that samples alternative interheme geometries and molecular conformations." Many of the transformed complexes were found to be more favorable for electron transfer than the initial complex based on a reduction in the iron-iron distance from the static model.

The cyt c-cyt peroxidase system was first modeled with the aid of computer graphics (Poulos & Kraut, 1980). A model was proposed based on visual optimization of the hydrogen bonding interactions between the complementary charged groups around the hemes. From this an electron transfer mechanism was proposed which utilized a system of π-π hydrogen bond interactions that form a bridge between the two hemes. The model was supported by chemical modification and kinetic data.

Analysis of this system was extended with Brownian dynamics (Northrup et al., 1988). As in the initial study, multiple plausible complexes were found. The complexes were found to result from "favorable electrostatic interactions" which subsequently led to "long-lived nonspecific encounters" and to specific encounters by rotational diffusion.

The association between three anti-lysozyme monoclonal antibodies and lysozyme was modeled six years later (de la Paz et al., 1986). Models were generated from crystal structures of immunoglobulin fragments and the cDNA sequences of Fv regions. The alpha carbon backbone of the structurally conserved framework regions were derived from and IgG myeloma protein. A model of one antibody and the corresponding lysozyme epitope was described after the full structure was energy minimized. The size and chemical nature of the predicted combining site correlated with epitope boundaries previously determined by affinity studies.

Modeling of protein-protein interactions is today a relatively new science and is continually being extended and improved. Recently, simulated annealing, which has been applied in crystallographic refinement, determination of the three-dimensional structures for inter-proton distance data, and conformational searches, has been applied to molecular docking (Yue, 1990). This promising method was applied to eight serine protease complexes with minimal cpu time when performed with distance constraints.

The goals of this thesis were to apply state-of-the-art computational and modeling methods to x-ray crystal structures of trypsin in an attempt to model the Michaelis complex of β-trypsin autolysis. The results of this modeling, used in conjunction with the vast literature on trypsin, and corroborated by empirical observations, offer new insights into this process and into the mechanism of action of calcium in slowing trypsin autolysis.

Methods

Computational

1. Linear Distance Plots (LDPs) of proteins were constructed utilizing alpha carbon coordinates of the crystal structures of proteins (Liebman, 1985a). Partitions of the alpha carbon backbone were created successively including five sequential alpha carbons. The sum, S, of the distances between the first alpha carbon (N terminus) of each partition and the remaining four is calculated. This value reflects the curvature of the virtually bonded alpha carbon chain within that segment. Partitions are created by sequentially moving along the alpha carbon backbone from the N-terminus to the C-terminus. A plot of the S values, the Linear Distance Plot, is generated containing (n-4) points, where n is the number of amino acids in the protein.

Classical elements of secondary structure such as alpha helices, beta sheets, and 3/10 helices have characteristic S values. LDPs have been shown to be useful for analyzing proteins by identifying components of secondary structure and defining their boundaries (Liebman, 1985a). Furthermore, it has been shown to be a method for comparing protein structures, irrespective of their amino acid sequence without requiring direct superposition, and for identifying regions of structural insertions and deletions (Liebman, 1985b).

Difference linear distance plot analysis (dLDP) is a method for the pairwise comparison of protein structures and identification of regions of local conformational changes. The dLDP is constructed by choosing one of the two proteins to be compared as the reference structure and subtracting the values of its LDP from that of the other protein. If the two proteins are identical, this difference will be 0.0 throughout, regardless of any

difference in their respective orientations. A plot of dLDP vs. residue number visually reveals regions of conformational difference (Liebman, 1986).

2. Structural superpositioning (SP), as discussed in this study, utilizes the topographical structural equivalencing procedure of Liebman (1985b) to obtain a statistically refined translation-rotation matrix based on the algorithm developed by Cox (Appendix of Muirhead et al., 1967). This algorithm minimizes the root-mean-square (rms) difference between sets of equivalenced atoms of molecules A and B. A rotation matrix, defined in terms of Eulerian angles, is obtained after first translating the centers of the individually orthogonalized center-of-mass coordinates of B onto A.

In the transformation the following steps take place:

a) the center-of-mass of molecule B is translated onto the center-of-mass of molecule A;

b) a procedure is applied which obtains the best rotation matrix by minimizing the merit function, F, where F is the rms difference between the positions of the equivalenced positions of molecules A and B.

c) atom pairs of the equivalenced set are eliminated by identifying outliers from the observed Chi-square distribution with three degrees of freedom. A conservative confidence level of 99.999% is used; and

d) the entire procedure (a-c) is applied iteratively until convergence has been achieved, that is, when outliers cannot be distinguished from the Chi-square theoretical distribution at the prescribed confidence level.

This analysis yields a single value metric, the root-mean-square deviation, as well as the distribution of the distances between all of the initially equivalenced atoms in the molecules. It is especially useful for evaluation of the global similarity between proteins but is capable of identifying regions of conformational variability.

3. van der Waal's (vdW) contacts were calculated by measuring interatomic distances between the two structures and accessing a 'look-up' table based on vdW radii of individual atoms. Atom-atom distances falling within the distances defined in the table (with addition of a 1 Å buffer to each atom because of hydrogen atom omission) were determined to be within van der Waals' contact, with the 1 Å buffer included. Below are the buffered distances (in Å) for the four heavy atom combinations of trypsin:

C-C	5.60	N-N	5.60	O-O	5.00
C-N	5.60	N-O	5.30	O-S	5.35
C-O	5.30	N-S	5.65		
C-S	5.65			S-S	5.70

4. Solvent accessible surfaces (sas), expressed as the accessible surface area divided by $4\pi R^2$ and multiplied by 100, were calculated by the method of Lee and Richards (1971) as implemented by Alexander Rashin. R is the sum of atomic radii and the radii of a water molecule. The values for these radii were taken from the data of Rashin (1984):

R (Å)	Atom type
1.4	oxygen; water
1.5	hydroxyl; terminal nitrogen
1.7	trigonal carbon; trigonal nitrogen
1.8	trigonal NH2
1.85	sulfur
2.0	tetrahedral carbon and nitrogen; sulfur with bound hydrogen

R and the atomic coordinates determine the surface on which the center of a water molecule makes contact with an atom of trypsin without penetrating any other atoms. The smooth outer surface contour constitutes the solvent accessible surface. It is calculated analytically and is made up of sections of spheres and tori that join at circular arcs.

5. The unit cells of two trypsin PDB entries were constructed in order to evaluate the effects, if any, of crystal environments on the conformational and thermal variabilities

of trypsin and its three physiological sites. One of four trypsin structures (1tpo) included in the library of Chapter 1 which crystallizes to space group P 21 21 21. 3ptn crystallizes to space group P 31 2 1.

The equivalent positions of the two space groups are:

P 21 21 21	P 31 2 1
x, y, z	x, y, z
1/2-x, -y, 1/2+z	-y, x-y, z+1/3
1/2+x, 1/2-y, -z	y-x, -x, z+2/3
-x, 1/2+y, 1/2-z	y, x, -z
	-x, y-x, 1/3-z
	x-y, -y, 2/3-z

The two crystal environments were generated by the following procedure:

a. transforming the orthogonal coordinates to fractional coordinates by applying the transformation matrix found in the respective PDB files;

b. computing the equivalent orientations by applying the appropriate symmetry operation;

c. computing all translational equivalents within two unit cells and checking for vdW contacts; and

d. transforming the unit cell components from fractional coordinates back to orthogonal coordinates.

An example of the calculations to arrive at the coordinates of the five 195S atoms of the 1tpo crystal is given below:

The orthogonal coordinates of CA195S in 1tpo1 (the PDB structure) and the other four generated molecules which make van der Waals contacts with 1tpo1 are:

1tpo1	0.330	12.527	23.242
1tpo2	-27.112	16.734	44.360
1tpo3	27.778	16.734	44.360
1tpo4	-0.333	-16.734	10.544
1tpo5	-0.333	41.786	10.545

The transformation matrix is:

$$T^{-1} = \begin{vmatrix} 1/54.89 & 0 & 0 \\ 0 & 1/58.52 & 0 \\ 0 & 0 & 1/67.63 \end{vmatrix}$$

$$= \begin{vmatrix} 0.01822 & 0 & 0 \\ 0 & 0.01709 & 0 \\ 0 & 0 & 0.01479 \end{vmatrix}$$

Converting 1tpo1 to fractional coordinates:

(0.330)(0.01822) = **0.0060**
(12.527)(0.01709) = **0.2141**
(23.242)(0.01479) = **0.3437**

The symmetry code for the P212121 space group, as noted above, is:

(a) x,y,z
(b) 1/2-x, -y, 1/2+z
(c) 1/2+x, 1/2-y, -z
(d) -x, 1/2+y, -z

Applying these symmetry operations results in the following fractional coordinates:

(a)	0.0060	0.2141	0.3437
(b)	0.4940	-0.2141	0.8437
(c)	0.5060	0.2859	-0.3437
(d)	-0.0060	0.7141	0.1563

Applying the inverse of T^{-1} to get back to orthogonal coordinates yields:

(a) x = (0.0060)(54.89) = 0.330
y = (0.2141)(58.52) = 12.527
z = (0.3437)(67.63) = 23.242

(b) x = (0.4940)(54.89) = 27.116
y = (-0.2141)(58.52) = -12.527
z = (0.8437)(67.63) = 57.059

(c) x = (0.5060)(54.89) = 27.774
y = (0.2859)(58.52) = 16.731
z = (-0.3437)(67.63) = -23.242

(d) x = (-0.0060)(54.89) = -0.330
y = (0.7141)(58.52) = 41.789
z = (0.1563)(67.63) = 10.571

The crystal environment of the 1tpo1 structure is arrived at by the following:

- 1tpo1 is (a).

- 1tpo2 has its x coordinate from (c) translated one negative unit cell length (27.116 - 54.890 = -27.112); its y coordinate from (c); and its z coordinate from (c) translated one positive unit cell length (-23.242 + 67.63 = 44.360).

- 1tpo3 has its x coordinate from (c); its y coordinate from (c); and its z coordinate from (c) translated one positive unit cell length (-23.242 + 67.63 = 44.39).

- 1tpo4 has its x coordinate from (d); its y coordinate from (d) translated one negative unit cell length (41.786 - 58.52 = -16.734); and its z coordinate from (d).

- 1tpo5 is (d).

The distances between the CA195S of the five structures (in Å) are:

1tpo1 -	1tpo2	35	1tpo2 -	1tpo3	55	1tpo3 -	1tpo4	55
	1tpo3	35		1tpo4	55		1tpo5	50
	1tpo4	32		1tpo5	50			
	1tpo5	32				1tpo4 -	1tpo5	59

Appendix A gives the packing analysis for the 1tpo1 (and 3ptn1) structures.

6. Residues were considered to be part of trypsin's active site if they met three criteria:

a. they had to make backbone vdW contacts with PTI in the 2ptc structure;

b. have at least 10% of the residue exposed to solvent in the native enzyme (see above); and

c. have more than five vdW contacts (see above) with the inhibitor.

Along with the 10 residues of the P5-P5' residues of the β-trypsin autolysis site and the 11 residues of the calcium binding loop (70-80), the active site is considered to be one of the three physiological sites in the analyses of Chapter 2. The 14 residues which meet these "active site" criteria are:

39Y	40H	41F	57H	96S	97N	98T
99L	192Q	193G	195S	215W	216G	219G

7. SCREEN is a fortran program which was developed to test the rotational space around the original configuration of the modeled trypsin autolysis dimers by measuring its overlap. The procedure involved translating the 'enzyme' and 'substrate' heavy atoms so that the C atom of the backbone carbonyl of the 'substrate' P1 residue was at the origin. The 'substrate' trypsin is defined as that which binds to the active site of the 'enzyme' and is hydrolyzed. The 'substrate' was then rotated in predefined intervals and ranges around the x, y, and z axes of the crystal coordinate system. Overlap between the two structures was determined by identification of all atoms of the 'enzyme' which were $\leq$ 2.7 Å to any atom of the 'substrate'. The cutoff distance of 2.7 Å was chosen based on analysis of the 2ptc crystal structure. In this complex between β-trypsin and PTI, only one heavy atom of the inhibitor was found to be within 2.7 Å (Lys15C of the inhibitor is 2.67 Å from the Ser195OG) of the enzyme.

'Cropped' structures are used in the autolysis dimer modeling (Chapter 3) and analyses of the 16 basic sites of trypsin as potential substrates in trypsin autolysis (Chapter 5). These structures had all sidechain atoms beyond CB removed for two reasons. First, the assumption was made that sidechain atoms beyond CB can adapt, at no energy cost, by rotation around the CA-CB bond and alleviate any steric overlap resulting from modeling of the dimers. This greatly facilitates the modeling. Second, this reduced the total number of atoms from 1629 to 1091 and reduced the cpu time needed to complete the calculations by approximately 50%.

8. QUANTA© is a software package which is the property of the Polygen Corporation (Waltham, Massachusetts). Used on hardware produced by Silicon Graphics, Inc., this program is a tool for molecular modeling and display. Several features of this

package were utilized in the display, analyses, and construction of models of β-trypsin association. Among these were: the "Torsion" command (which allows manipulation and storage of backbone and sidechain torsional angles); features which produced stick figure and van der Waals representations; performed rotations and calculated distances; and evaluated neighbors and hydrogen bonding patterns.

9. CHARMM is a molecular mechanics and dynamics program (Brooks et al., 1983) which was employed to remove steric overlap in the modeling of the autolysis dimers. Each minimization was performed with only polar hydrogens (hydrogens bonded to oxygens or nitrogens) on both the 'enzyme' and 'substrate'. Other atoms were treated as 'united atoms'. The force field utilized was that incorporated into the program for standard proteins (Brooks et al., 1983). The steepest descents methods was employed to a maximum of 10,000 steps or until the energy tolerance of 0.1 kcal was satisfied. The calcium potential employed was that developed by Hori et al. (1987).

10. Calculations of linear-least-squares regression fits and sample correlation coefficients were performed with a fortran program based on that published by Isenhour and Jurs (1979). Regression analysis indicates to what degree variables are related while correlation analysis yields a number which is an index of how closely two variables move together. Minor modifications to the published program included tailoring of input and output statements and renaming of x and y variables. All regressions were performed with equal weights to all point.

Experimental

Purified β-trypsin was isolated according to the method of Schroeder and Shaw (1968). Stock trypsin (Type I) was obtained from Sigma Chemical Company. 300 mg was dissolved in 0.1 M Tris, 0.02 M $CaCl_2$, 1 mM benzamidine at pH 7.1 and was subjected to anion exchange chromatography. The 5 cm diameter SP-Sephadex column used was 40 cm in length. Pooled β-trypsin fractions were then lyophilized and stored at -10° C. Figure 5.1 shows the elution pattern of a representative purification.

To eliminate the effects of autolysis during collection of the infrared spectra, calcium binding was studied using β-trypsin inhibited with two active site inhibitors, diisopropylfluorophosphate (DIFP) and benzamidine (BA). DIP-trypsin was prepared by the method of Cunningham (1954). BA-trypsin spectra were collected in 2 mM benzamidine. Although atomic absorption analysis indicated that less than 0.5 ppm calcium was present in the dried protein samples (Thompson, 1990), all spectra collected had 1 mM ethylene glycol bis (β-amino ethyl ether) tetra-acetic acid (EGTA) added to complex any trace amounts of calcium which may have been present in the buffers.

Fourier-transform infrared spectra were collected at room temperature under dry nitrogen with a Nicolet 740 SX FTIR system on 3% (w/v) trypsin samples in either 20 mM acetate (pD 5) or 20 mM imidazole (pD 6.9) at the Eastern Regional Research Center (ERRC) of the United States Department of Agriculture in Philadelphia, PA. Buffers were prepared in D_2O and the pD was determined by adding 0.4 to the pH reading (Covington et al., 1968) of a Horiba, Cardy glass electrode pH meter. Stock solutions of 1 M $CaCl_2$ and 50 mM benzamidine•HCl in D_2O were added to the trypsin solutions to bring the final concentrations to 20 mM and 1 mM respectively. Spectra were recorded in IR cells which had CaF_2 windows and teflon spacers (pathlength = 75 μ).

Spectra were recorded to a resolution of 2 cm^{-1} by coadding 4096, double-sided interferograms. The interferograms were Fourier-transformed after application of a Happ-Genzel apodization function (Griffiths & DeHaseth, 1986):

$$A(\delta) = 0.54 + 0.46 \cos(\pi\delta/\Delta)$$

δ = mirror position
Δ = maximum retardation of mirror

Spectral contributions from residual water vapor in the light path were subtracted with factors determined by subtracting the second derivative spectrum of water vapor and the buffer from that of the trypsin sample. Spectral contributions for buffers were subtracted directly for absorbance spectra. The water vapor in the light path was subtracted by varying the subtraction factor until the nonabsorbing region from 1700-1800 cm^{-1} appeared featureless. This method results in a more reliable correction that the subtraction of the original absorbance spectra because sharp vapor lines are amplified by the derivatizations and may have been invisible in the original spectra. In an analogous fashion, spectral contributions from the buffers were subtracted by varying the subtraction factors until the 1725-1900 cm^{-1} and 3800-4000 cm^{-1} regions appeared flat. The subtraction factor for the buffers was generally found to be 0.90 - 0.95.

Derivative spectra, calculated analytically (Susi & Byler, 1983) with Nicolet FTIR software, Version 4.3, allowed visualization of individual component bands in overlapped regions. For the two forms of inhibited trypsin studied, second derivative spectroscopy enabled the estimation of the initial peak positions* to be (in cm^{-1}):

1624 1633 1644 1655 1663 1674 1683 1694 1699**

* variability in the final peak positions has been found to be within a range of 0.1 - 0.25 cm^{-1}, based on initial peak position estimates of $\pm$ 2 cm^{-1} [S. Prestrelski, personal communication]

** this peak was only found in the BA-trypsin form

Spectral deconvolutions of the amide I region were performed by mathematical manipulations in the Fourier domain (Kaupinnen et al., 1981) with the use of the Nicolet software. Optimal deconvolution of the spectra was performed by estimating the band widths of the component bands in the original spectrum to be 18 cm^{-1} (full-width at half-height). Values of 10 - 25 cm^{-1} have been previously used for the amide I region of proteins (Byler & Susi, 1886; Susi & Byler, 1986; Surewicz & Mantsch, 1988). A resolution enhancement factor, which is approximately the ratio of the original band width to the deconvoluted band width, of 2.8 was found to be optimal for all spectra, and is identical to the value chosen for previous trypsin studies (Prestrelski et al., 1991). This value was optimized by starting with a low value and systematically increasing it until periodic noise appeared in spectral regions where no absorbance existed. The value chosen then is the highest with no noise. Its maximal value thus ultimately depends on the signal-to-noise ratio of the original spectra. The Fourier self-deconvolution method has previously been shown to not change component band areas (Kauppinen et al., 1981).

To obtain the integrated intensities of the component bands, least-squares curve fitting was performed with an iterative Gauss-Newton non-linear regression program (Byler & Susi, 1986). Curve fitting was done on deconvoluted spectra with initial estimates of the individual positions, heights, and widths of the bands that are varied. Peak positions, taken from the second derivative spectra, were the same as those identified above. The half-heights were estimated to be 0.1 absorbance units and the half-widths were estimated to be 3 cm^{-1}. Curve fitting results on trypsin spectra were previously found to be independent of these estimates [S. Prestrelski, personal communication]. The deconvoluted spectra were fitted directly with Gaussian band profiles as previously described (Pierce et al., 1990). Finally, relative areas of the component bands in the amide I' region were obtained by dividing the component by the total amide I area.

Chapter 1
Library of Conformational and Thermal Variabilities

Goals

To establish a database of alpha carbon conformational variability of proteins resulting from perturbation in chemistry and environment (not due to evolutionary differences or significant chemical modifications) and to ascertain whether or not trypsin has any attributes which distinguish it from other proteins.

1.1 Library Construction

To establish a library of conformational and thermal variabilities among independent protein structures deposited in the Protein Data Bank, several steps were undertaken. First, one structure, the 'reference molecule' was chosen from a protein set which has multiple structures. The reference molecule, which was compared in a pairwise manner to the remainder of the protein structure set, was typically selected to be the native, uncomplexed structure. When no such 'native' structure exists, a structure bound by a small, organic ligand was chosen.

Second, the role that refinement methods (and starting structures) have on the coordinates deposited by the investigators was assessed. The crystallographer's need to use an estimated starting structure in diffraction pattern analysis potentially limits the number of independent observations in the PDB by biasing the statistical data to values which reflect an unnatural homology.

The influence of the refinement methods on the determination of structures is exemplified in a number of cases. To test the effect of this influence on the dLDP analysis and SP results, and to assess its significance on construction of the library, comparisons

were performed between structures which had been obtained by two or more different refinement procedures and between structures in which one had not been refined at all.

In the first six comparisons listed in Table 1.1, a refined structure was compared to one that was not refined. In the remaining nine comparisons, the two structures were refined by different methods.

These results show that refinement does have a significant effect on the final structures. In the comparisons involving one structure which had not been refined, the variability is quite large, with SP rms deviations over 1 Å in three of the five cases. Therefore, no protein was included in the library that had not been refined. In cases where more than one structure, resulting from different refinement procedures, exists, the most recent version deposited in the PDB was used.

Closely connected to assessing the influence of refinement on the final determined structures is assessing the effect that the precision and accuracy of the atomic coordinates have on the structural comparisons. The accuracy of the coordinates, which reflect the agreement between the reported crystal structure and the biological conformation of the protein, is a function of the systematic errors in the crystal structure analyses. Treatment of it is currently an active area of research. However, the observations that the three dimensional structure observed in the crystal is reproducible within a given family, and is independent of organism or its position within the unit cell, suggests that the coordinates closely represent the true three-dimensional structure of these proteins. Superimposed on this experimental background noise are significant changes which were shown to be induced by a number of chemical and environmental forces.

Precisions of the coordinates and their correlation to resolution were assessed by calculation of the alpha carbon - alpha carbon (CA - CA) distances for all adjacent, non-proline, amino acids. Column 5 of Table 1.2 gives the means and standard deviations of these distances for each protein included in the comparison library.

The range of the average CA - CA distances for the 79 protein structures used in the library comparisons is 3.76 to 3.82. With only a few exceptions, entries have a standard deviation less than 0.10 Å. The least squares test (see Table 1.3) shows no correlation between the precision of the two structures (defined here as one standard deviation of the CA - CA distances) and their resolution. Thus, no comparisons were excluded based on the precision of the alpha carbon coordinates.

Fifth, the reported resolution of the individual structure was considered. No comparison was performed using a structure with a resolution of worse than 2.9 Å. The stated resolutions of the proteins which comprise the library are divided into two groups, with 2.2 Å being the separating resolution (see Table 1.2). 74 of the 86 structures (86%) are solved to 2.1 Å or better, with the remaining 12 being solved to 2.1 to 2.9 Å. The seven complexed and uncomplexed trypsin structures are all solved to 2.1 Å or better, with the five uncomplexed structures all solved to at least 1.7 Å.

The analysis of refinement effects mentioned above, along with observations of all other structure comparisons of the PDB, led to the choice of an initial SP fit of 0.10 Å or higher for inclusion in the database. A comparison with a initial SP fit of 0.10 Å or better was considered to be unduly influenced to an unnatural homology, by whatever means, and thus was not included in the library. This criterion, along with the five outlined previously, eliminated about 100 comparisons. Of the approximately 160 possible protein comparisons of structures deposited in the PDB as of January 1, 1987, only 56, (35%) were included in the library.

Finally, statistical analyses and application of the 56 comparisons among 26 types of proteins was conducted to construct a library of conformational changes in proteins with identical primary sequences. Changes were indicated by the method of difference linear distance plot (dLDP) analysis and the results were contrasted and compared with the superpositioning (SP) method.

1.2 Results

1.2.1 Correlations to Resolution

Two correlations of potential significance to this study were examined and are reported in Table 1.3. Both alpha carbon precision and structural equivalence are shown to be uncorrelated to the average resolution of the two structures under comparison based on at least one of three observations: 1) the correlation coefficient is close to 0.00; 2) the slope of the regression line is 0.0; or 3) the slope of the regression line and/or the correlation coefficient is lowered or reversed when 'selective inclusion' of one representative from each group is chosen.

'Selective inclusion' entailed reducing the library to a smaller set consisting of only one comparison from each group - based on it having either the lowest or highest initial SP rms deviation of the group. For example, in the four comparisons of the chymotrypsin structures, the initial SP rms deviations are 0.60, 0.42, 0.46, and 0.49 Å. Selective inclusion utilized the value of 0.42 Å in the 'lowest' correlation test and 0.60 Å in the 'highest'. This was done for all 26 groups which comprise the library.

If this procedure results in diminution or reversal of the sign of the slope of the regression line and/or significant lowering of the correlation coefficient, it indicates that the results are dependent upon the comparison which is chosen to represent the group. Selective inclusion was performed to test whether groups with large numbers of comparisons, inordinate precisions, or anomalous structural equivalence have biased the correlation tests.

In Table 1.3 it is shown that no correlation exists between the reported resolutions of the structures under comparison and their precision (defined as one standard deviation of the CA - CA average distance). The bases for this conclusion are that the slope of the regression line is small (it also has a large standard deviation) and its correlation coefficient is small (0.02).

Table 1.3 also shows the relationship between the average reported resolution of the two structures being compared and the results of dLDP analysis and initial and final SP fits. Selective inclusion reverses the slope of the regression lines here. In the 56 comparisons of the entire library, it could be concluded that there is a slight correlation between average resolutions and the results of the two methods (r values of 0.26, 0.25, and 0.21 respectively). But selective inclusion of the 'lowest' and 'highest' comparisons reverses the sign of the slope of the least squares line - indicating that the correlation is dependent on the comparison chosen to represent each of the groups. It is therefore concluded that there is no correlation between average reported resolutions and dLDP and SP results for the comparisons which comprise the library.

1.2.2 Alpha Carbon Conformational Variability of the Library

Table 1.4 summarizes the SP and dLDP alpha carbon structure comparisons of the library. The four citrate synthase structures exhibit the most structural variability of all of the proteins which comprise the library. Other proteins with large variability are the conconavalins, the hemoglobins, the lysozymes, and the pancreatic trypsin inhibitors.

Several comparisons have lower initial SP rms deviations than the trypsins. The most notable are one of the cytochromes, the leghemoglobins, and the plastocyanins. A general trend is that the smaller proteins tend to have smaller variability. The exceptions to this are lysozyme and pancreatic trypsin inhibitor.

Both methods identify the five trypsins as being highly structurally homologous. The initial SP rms deviations and number of dLDP outliers are both well below the median of the entire library. The comparisons between the two trypsin/PTI complexed structures also show similar homology. The structural variability of the trypsins is also shown to fall between that of two proteins of similar molecular weight. Conconavalins show a larger variability while the thermolysins are lower. Finally, the two other serine proteases which comprise the library, chymotrypsin and kallikrein, have larger variabilities than trypsin.

1.2.3 Space Group Effects

Fourteen of the 56 comparisons of the library involve structures from different space groups. Different crystallographic space grouping of the same protein is usually the result of different chemistry. These 14 comparisons are: 3cyt-4cyt, 1cts-2cts, 1cts-4cts1, 1cts-4cts2, 2hhb-1hhoa, 2hhb-1hhob, 2hhb-2hcoa, 2hhb-2hcob, 2mhb-2dhb, 1lzt-2lyz, 1lzt-1lym1, 1lzt-1lym2, 1rn3-1rsm, and 1tpo-3ptn. The average initial SP rms deviation of this group is 1.1 Å, with the lowest rms deviation being the 1tpo-3ptn comparison (0.26 Å) and the highest being the 1cts-2cts comparison (2.4 Å). All three of the citrate synthase comparisons have very large initial SP rms deviations. When these three comparisons are discarded, the average deviation of the remaining 11 comparisons is reduced to 0.74 Å. This is still a large SP rms deviation relative to the entire library and indicates that structures derived from crystals of different space groups show more variability than those derived from the same space group. dLDP analysis confirms this observation - only one of the 14 comparisons (1rn3-1rsm) has no outliers and the remaining 13 have up to 23.6% (1cts-4cts1) of their alpha carbons identified as outliers.

1.2.4 Structural and Thermal Parameter Correlations to Distance from Center of Mass

Table 1.5 compares the relationship between the absolute value of dLDP magnitudes*, initial and final SP rms deviations, and B values to the distance from the center of mass (d) of the proteins being compared. It is seen that B values exhibit greater correlations to d than do the two other parameters. The B value correlations to d range from 0.06 to 0.78 for all proteins of the library and all are positively related. The average correlation of the B values to d is 0.42. The average correlation of the initial SP rms deviations to d is 0.30 and the absolute values of the dLDP magnitudes to d is only 0.08.

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* Note: dLDP magnitudes (the absolute values of) were used for the determination of correlations here. This is in contrast to the percentage of outliers from the dLDP analysis in Table 1.3.

Analyses of the dLDP magnitudes and initial SP rms deviations as a function of d also examines the dependence of the two methods on distance to center of mass of the protein and allowed a comparison of their correlation to that of the B values. Several themes are revealed in these analyses. First, as shown above, both conformation comparison methods exhibit correlations to d considerably lower than those of the B values. Second, only two of the correlations of the SP method are negative (the first two leghemoglobin comparisons, llh1-1lh2 and 1lh1-1lh3). In contrast, 20 of the 56 dLDP correlations are negative. Third, 51 of the 56 SP rms correlations are larger in magnitude than the corresponding dLDP correlations (the six exceptions being the calcium binding parvalbumin, a leghemoglobin, a myoglobin, the phospholipase, and one of the thermolysin comparisons).

Since the B values and SP rms deviations are both positively correlated to d in 54 of the 56 comparisons, the B values must necessarily be positively correlated to the SP rms deviations in those 54. Conversely, the 20 dLDP comparisons with negative correlations to d will have negative correlations to the B values. This indicates that the dLDP method does not identify surface residues as outliers to the same extent as the SP method.

1.2.5 B Value Relationships

In Table 1.6 are tabulated the reported B values of the structures included in the library. Ten of the 79 structures used in comparisons have no B values reported by the authors. Five of these structures have resolutions better than 2.1 Å. Two other PDB entries (1cpv and 2lyz) have B values reported as constant throughout the structure (9.0 and 5.0 respectively). Six others (2mhb, 2lyz, 1mb5, 2mbn, 3mbn, and 1rn3) have B values far removed from the rest of the library, with averages below 2.0.

After removing these 18 structures, a correlation test was performed between the resolution and average alpha carbon B values for the structures. The correlation coefficient was found to be 0.08 - indicating that no correlation exists between resolution and the average of alpha carbon B values.

The highest B values are found in the hemoglobin structure, 1hho. Its average alpha carbon B value is 35.2. For comparison, the 61 structures of the library have an average alpha carbon B value of 14.8. Other library structures with inordinately high B values include all four citrate synthase structures, one of the complexed trypsin structures (2ptc), and one of the uncomplexed trypsin structures (3ptp).

The five comparisons between two independently reported structures of the asymmetric unit have B values consistent with the fact that they are in approximately the same environment. Only kallikrein shows alpha carbon B value averages which differ to any appreciable amount - about 20%. The cytochrome structures have alpha carbon averages within 5% of each other, while the two chymotrypsin and the citrate synthase comparisons have mean values within 2% of each other.

1.3 Discussion

The library was constructed and analyzed for information on alpha carbon conformational variability and thermal parameter properties of a variety of crystalline proteins and for specific assessment of bovine β-trypsin. The PDB is composed predominantly of soluble, globular proteins, with only one membrane-bound structure having been solved by January 1, 1987. A membrane-bound bacterial photosynthetic reaction core has been solved (Deisenhofer, 1985) and recently deposited in the PDB. The library constructed is comprised of 11% hemoglobin and 9% trypsin structures. Even so, this form of analysis probably does not preclude generalizations about other soluble proteins and other groups of the PDB not included in the library.

The role of the refinement procedure in X-ray crystal structure determinations was assessed before construction of the library. Refinement procedures were shown to have a significant effect on alpha carbon positioning. In the case of subtilisin and alcohol dehydrogenases, where a refined structure was compared to one that had not been refined

at all, the rms deviations were over 1 Å. These observations necessitated the decision to exclude structures that had not been refined.

The variability in the nine comparisons between structures solved by different refinement methods is in close agreement with the variability found by several other investigators. The range here is 0.04 - 0.40 Å and the average is 0.17 Å. Chambers & Stroud (1979) attributed the difference of 0.15 - 0.20 Å in two non-isomorphous crystal structures of trypsin to be predominantly the result of errors introduced during the refinement procedures. Similarly, Bode & Schwager (1975b) estimated standard deviations of their trypsin coordinates to be about 0.1 Å, Janin & Chothia (1976) determined the rms movement of all trypsin atoms to be 0.134 Å during the refinement procedure, and Huber et al. (1974) estimated the deviation in their trypsin atom coordinates to be 0.15 Å. Shanaan (1983) found in hemoglobin that three cycles of unrestrained refinement, run to check the effects of restraints on the 1hho final structure starting from the final model, yielded a rms deviation of the main chain atoms of 0.12 Å.

This analysis supports the liberal criteria of 0.1 Å as the minimum SP rms deviation for inclusion in the library. A more conservative cutoff would have reduced the number of comparisons (see below). Any comparison which yielded a rms deviation below 0.1 Å was considered to result from a comparison of the same structure - an assertion supported by the refinement method analyses.

The distribution of the reported resolutions of the structures comprising the library shows that 80% of the structures are at resolutions of 2.05 Å or higher. The variation within individual protein sets does not typically reflect this same range in resolutions. For example, resolutions of all trypsins range from 1.4 to 1.7 Å, while resolutions of all of the serine proteases range from 1.4 to 2.1 Å. This compares closely to the trypsin comparison study of Marquart et al. (1983) on only structures with resolutions of 1.9 Å or better.

Structures solved to 2.9 Å were utilized for several reasons, even though the inclusion of structures of lower resolution theoretically introduces more random and

systematic error (Glusker, 1985). First, the reported data resolutions in the PDB are defined ambiguously. Second, no correlation was found between resolution and precision, SP rms deviations, and percentage of dLDP outliers. This is probably because refinement procedures frequently correct bond length distortions with high fidelity - a contention which is supported by the consistency in precisions amongst the structures of the library. Third, as always, there was a trade-off between sample size and selection criteria. A cutoff of 1.9 Å, for example, would have reduced the total number of comparisons to 25 and would have excluded 14 entire sets of comparisons.

The dLDP comparisons resulted in determination of a dLDP value of 1.63 standard deviations of the entire library considered to reflect significant changes to the backbone conformation of a protein. The rationale for the choice of 1.63 standard deviations of the dLDP values was that this value identified roughly the same percentage (7.2%) of alpha carbon outliers as the SP method (which rejects outliers based on a theoretical Chi-square distribution with three degrees of freedom - see Methods). The identity of the actual outliers, however, differs significantly - only 26% of the outliers determined by dLDP analysis are also identified as outliers by the SP method. This indicates that the dLDP method may be useful for identifying heretofore unknown variability.

Both the SP and dLDP methods yield a bimodal distribution of comparison results. Approximately one third of the comparisons, exhibiting no outliers by dLDP analysis and an initial SP rms deviation of less than 0.3 Å by the SP method, can be interpreted as being indistinguishable within the limits of discriminating differences on a structural basis alone. The disparity in the group of 1/3 could be partly the result of errors introduced in the refinement process. The results of the test of different refinement methods support this statement - an rms deviation of 0.3 Å is about twice the variability found in structures solved by different refinement procedures.

The comparisons between structures from the same asymmetric unit and from different space grouping indicate the extent that environment can influence backbone

conformations. The 5cha, 3cyt, 4cts, 2pka, and 1lym structures all have two molecules in the asymmetric unit and have initial SP rms deviations of about 0.45 Å. The alpha carbon variability of the different space group comparisons (excluding citrate synthase) is greater than this - 0.74 Å. For comparison, Chothia & Lesk (1986) estimated the contribution to the SP rms deviation from experimental error and different molecular environments to be 0.33 Å. This was based on comparisons of five structures (trypsin inhibitor, tuna cytochrome c, azurin, rat protease, and deoxy hemoglobin) determined in either different space grouping or in crystals with more than one molecule per asymmetric unit.

These variations are all above any variability found in the trypsin structures. The largest deviations of this group are in the PTI-inhibited structures - at 0.3 Å. The 1tpo-3ptn comparison involves structures from two different space groups and is only 0.26 Å. This indicates that trypsin's backbone conformation is preserved in different environments and is relatively more stable than other proteins of the PDB.

Of the 26 groups of proteins compared, citrate synthase shows the most structural variability while the leghemoglobins and plastocyanins show the least. Being outliers of the overall distribution, these groups naturally merit more detailed examination.

In the case of citrate synthase, three of the four structures have lower resolutions (2.7, 2.9, and 2.9 Å) and all are of different space groups. These two factors together could be contributing to the large variability in conformations of the structures as identified by both methods. However, it is truly the physical response of the enzyme to cofactor and substrate binding which results in a large range of motion. This enzyme has been extensively analyzed (Wiegand et al., 1984) and has been reported to consist of domains which are connected through hinge regions. It is the enzyme's response to cofactor and ligand binding which leads to the large backbone changes. The molecule is a dimer of identical subunits composed of a large and a small domain. It is predominantly α-helical, with 40 helices per dimer packed tightly to form a globular protein. The tetragonal form is the "open configuration" with a deep cleft between the two domains, while the monoclinic

form is "closed". An 18° rotation of the small domain relative to the large domain closes the open form to shield bound citrate from solvent with the cysteamine part of coenzyme A (Remington et al., 1982).

At the other extreme, the leghemoglobins and plastocyanins show very high structural homology (even across seven independently reported structures). The leghemoglobin structures are multiple deposits from the same investigators, but could not be excluded based on the criteria outlined earlier for incorporation into the library. The initial SP rms deviations of the 11 comparisons from the two groups range from 0.12 to 0.21 Å (close to the 0.10 Å selection criterion limit). Furthermore, dLDP outliers were identified in only two of the leghemoglobin comparisons (1lh1-1lh6 and 1lh1-1lh7) while all five plastocyanin comparisons have none. This preservation of conformation could be related to the function of these molecules. Both are involved in electron transport and are small globular proteins composed of a single domain. Preservation of a critical conformation under different physiological conditions could be necessary for maintenance of the electron transport properties of these important proteins.

The trypsin comparisons fall into the category of structures of high homology. This is surprising considering the different environments and conditions that trypsins have been crystallized under - especially the complexation with PTI. Trypsin possesses only two α-helices and two deformed β-barrel domains, as revealed by inspection of the DM (Liebman, 1986). The structure of the enzyme apparently evolved to remain constant over a wide range of conditions. This makes sense if one considers that it is secreted into the intestine and must act on proteins under a variety of environmental conditions.

The underlying structural data of the library is not, and should not be, normal and bivariate because the observations which produce the crystallographers' three-dimensional coordinates are not always independent. Although this was attempted to be minimized in the library construction, this means that product-moment correlations are only valid to convey general relationships and are thus 'descriptors'.

B Values show the greatest correlation to distance from center of mass of the three parameters tested. Furthermore, all of the B v. d relationships have positive slopes, consistent with the analysis of Petsko and Ringe (1984), in which they showed that B values are, in general, larger for residues on the surface of proteins. Moreover, these surface residues are usually polar and charged amino acids. All of these observations are consistent with a view of a protein possessing a dynamic surface and stable core - features which are very likely important for structural integrity and biological function. The trypsin structure B value attributes were all shown to be typical of the other proteins of the library.

The observation that the SP rms deviations are positively correlated to d in 54 of the 56 comparisons can be explained in two ways: 1) it can be construed as evidence that the core of proteins are less flexible and more structurally similar than the solvent accessible surface (the high correlation of the thermal parameter B, to d would support this); and/or 2) it can be a result of the dependence of the SP results on distance to the center of mass of the protein and the concomitant distribution of atom centers as a function of d. The significance of the second point is dramatized by the dLDP analysis of the 56 comparisons. This method found 21 negative correlations to d. This is evidence that the dLDP method does not identify residues on the protein surface as outliers to the same extent as the SP method, and again indicates that it may be useful for identifying heretofore unknown variability.

Dickerson et al. (1971) succinctly summarized the properties and response of soluble proteins, before extensive crystal structures had been determined, when he described them as such:

> " . . . Globular proteins in general appear to be, not rigid, and not floppy, but elastic. They have a unique minimum energy conformation to which they will return after perturbation if permitted to do so, but the energy required to deform them temporarily without breaking covalent bonds or a significant number of hydrogen bonds is small. Resilience is apparently more advantageous in a protein molecule than either brittleness of plasticity."

Conclusion: The conformational variability and thermal parameter results of the crystal library indicate that both are correlated with distance from the center of mass of the proteins. Trypsins were found to be structurally homologous molecules under a variety of conditions. Trypsin possesses no attributes which distinguish it from other globular proteins of the Protein Data Bank. Furthermore, no evidence exists which indicate that it is capable of undergoing large conformational changes in response to ligand binding.

Table 1.1

Refinement Method Effects

Initial = initial SP rms deviation
Final = final SP rms deviation
No. = number of outliers determined by the SP method
dLDP = number of outliers determined by the dLDP method

4adh-5adh (374aa) [v. none]
Initial = 0.52 Final = 0.24 No. = 25 dLDP = 26

4adh-6adh1 (374aa) [v. none]
Initial = 1.33 Final = 1.19 No. = 14 dLDP = 94

4adh-6adh2 (374aa) [v. none]
Initial = 1.37 Final = 1.25 No. = 11 dLDP = 113

2est-1est (240aa) [v. none]
Initial = 0.24 Final = 0.22 No. = 8 dLDP = 3

2lyz-1lyz (129aa) [v. none]
Initial = 0.38 Final = 0.36 No. = 2 dLDP = 14

1sbt-2sbt (275aa) [v. none]
Initial = 1.05 Final = 1.01 No. = 4 dLDP = 118

7cat-8cat (498aa) [v. relaxed symmetric constraint]
Initial = 0.12 Final = 0.12 No. = 7 dLDP = 0

2hhb-3hhb (287aa) [Jack & Levitt v. symmetry avgd. + Levitt method]
Initial = 0.15 Final = 0.13 No. =17 dLDP = 0

2hhb-4hhb (287aa) [v. unrestrained refinement]
Initial = 0.15 Final = 0.13 No. = 16 dLDP = 1

2mbn-1mbn (153aa) [Diamond + Fourier Method v. Diamond Method]
Initial = 0.56 Final = 0.40 No. = 8 dLDP = 19

2lyz-3lyz (129aa) [Model Building + Diamond Method v. Diamond + lowered filter level]
Initial = 0.10 Final = 0.07 No. = 8 dLDP = 0

-4lyz [v. Diamond method. Electron counts refined in a separate step]
Initial = 0.28 Final = 0.25 No. = 4 dLDP = 1

-5lyz [v. Diamond method. Atomic radii refined in a separate step]
Initial = 0.28 Final = 0.25 No. = 4 dLDP = 1

-6lyz [v. Diamond method with additional refinement of electron counts]
Initial = 0.15 Final = 0.12 No. = 9 dLDP = 0

1lh1-2lh1* (153aa) [v. k=0.0000 (crystal part of residual 1/10 energy part)]
Initial = 0.04 Final = 0.04 No. = 0 dLDP = 0

* Note: all 7 of the leghemoglobin structures had this additional structure. Only one is shown with the range of the SP deviations being 0.04-0.06 Å in the entire group.

Table 1.2

PDB Entries Comprising the Library

(CA-CA) = alpha carbon - alpha carbon distance mean and standard deviation

PDB Code	Protein Description	Resolution (Å)	Space Group	(CA - CA)
4atc	Aspartate Carbomoyl Transferase	2.6	P 3 2 1	3.79±0.05
5atc	/CTP bound	2.8	P 3 2 1	3.79±0.05
5cha1	alpha-Chymotrypsin(Bovine)	1.67	P 2 1	3.81±0.07
2cha	(Tosylated)	2.0	P 2 1	3.81±0.04
4cha1	(mol. 1 of asymmetric dimer)	1.68	P 2 1	3.80±0.03
4cha2	(mol. 2 of asymmetric dimer)	1.68	P 2 1	3.80±0.03
5cha2	(mol. 2 of asymmetric dimer)	1.67	P 2 1	3.80±0.03
2cna	Conconavalin A (Canavalia Ensiformis)	2.0	I 2 2 2	3.80±0.00
3cna	(Canavalia Ensiformis)	2.4	I 2 2 2	3.80±0.01
1cpv	Calcium-binding Parvalbumin (Set 6A)	1.85	C 2	3.80±0.00
2cpv	(Set 6H)	1.85	C 2	3.79±0.19
3cyt1	Cytochrome C (Albacore, oxidized)	1.8	P 43	3.77±0.05
3cyt2	("inner" molecule of oxidized form)	1.8	P 43	3.76±0.05
4cyt	(Albacore reduced)	1.5	P 21 21 2	3.79±0.03
2c2c	Cytochrome C (oxidized)	2.0	P 21 21 21	3.81±0.05
3c2c	(reduced)	1.68	P 21 21 21	3.82±0.07
1cts	Citrate Synthase (Pig)	2.7	P 41 21 1	3.78±0.05
2cts	(COA, citrate complex)	2.0	C 2	3.78±0.05
4cts1	(oxaloacetate complex, mol. 1)	2.9	P 43 21 2	3.78±0.07
4cts2	(mol. 2 of aymmetric dimer)	2.9	P 43 21 2	3.78±0.07
3fxn	Flavodoxin (Clostridium MP, oxidized)	1.9	P 31 2 1	3.78±0.04
4fxn	(reduced)	1.8	P 31 2 1	3.79±0.04
2hhb	Hemoglobin (Human, deoxy)	1.74	P 21	3.80±0.04
1hho	(oxy)	2.1	P 41 21 2	3.77±0.06
2hco	(carbonmonoxy, NRG refined)	2.7	P 41 21 1	3.78±0.02
2mhb	Hemoglobin (Horse, aquo met)	2.0	C 2	3.79±0.01
2dhb	(deoxy)	2.8	C 2 2 21	3.85±0.05
1hmq	Hemerythrin (Met)	2.0	P 4	3.81±0.07
1hmz	(Azido, Met)	2.0	P 4	3.81±0.08
2pka1	Kallikrein A (Porcine)	2.05	P 41 21 2	3.78±0.02
2pka2	(mol. 2 of asymmetric dimer)	2.05	P 41 21 2	3.79±0.04

Table 1.2 (cont'd)

PDB Code	Protein Description	Resolution (Å)	Space Group	(CA - CA)
1lh1	Leghemoglobin (Acetate, Met)	2.0	B 2	3.82+0.16
1lh2	(Aquo, Met)	2.0	B 2	3.82±0.15
1lh3	(Cyano, Met)	2.0	B 2	3.82±0.15
1lh4	(Deoxy)	2.0	B 2	3.82±0.16
1lh5	(Fluoro, Met)	2.0	B 2	3.82±0.15
1lh6	(Nicotinate, Met)	2.0	B 2	3.82±0.17
1lh7	(Ferro)/Nitrobenzene	2.0	B 2	3.82±0.16
1lzt	Lysozyme (Hen Egg-white, Triclinic)	1.97	P 1	3.82±0.14
2lyz	(Set RS5D)	2.0	P 43 21 2	3.80±0.01
7lyz	(Triclinic)	2.5	P 1	3.81±0.02
1lym1	(Monoclinic)	2.5	P 21	3.83±0.08
1lym2	(mol. 2 of asymmetric unit)	2.5	P 21	3.83±0.08
1mbd	Myoglobin (Sperm Whale, Deoxy)	1.4	P 21	3.81±0.06
2mbn	(Met)	2.0	P 21	3.80±0.03
3mbn	(Deoxy)	2.0	P 21	3.80±0.03
1mbo	(Oxy)	1.6	P 21	3.80±0.04
1mb5	(CO, Neutron)	1.8	P 21	3.80±0.06
1bp2	Phospholipase A2 (Bovine)	1.7	P 21 21 21	3.80±0.03
3bp2	(Transaminated)	2.1	P 21 21 21	3.81±0.07
9pap	Papain (Cys-25 oxidized)	1.65	P 21 21 21	3.81±0.03
1ppd	(2-hydroxyethylthiopapain)	2.0	P 21 21 21	3.80±0.02
1pad	Papain D	2.8	P 21 21 21	3.80±0.01
1pcy	Plastocyanin (Cys-25 oxidized)	1.6	P 21 21 21	3.79±0.02
2pcy	(Apo, pH 6)	1.8	P 21 21 21	3.80±0.02
3pcy	(Hg+ substituted)	1.9	P 21 21 21	3.80±0.02
4pcy	(pH 7.8)	2.15	P 21 21 21	3.79±0.02
5pcy	(pH 7.0)	1.8	P 21 21 21	3.80±0.02
6pcy	(pH 3.8)	1.9	P 21 21 21	3.80±0.02
1rn3	Ribonuclease A	1.45	P 21	3.79±0.03
5rsa	(X-ray + Neutron)	2.0	P 21	3.80±0.08
6rsa	(Uridine-Vandate)	2.0	P 21	3.80±0.08
1rsm	(Lys 7-DNP-/Lys 41)	2.0	P 21 21 21	3.81±0.03
4rxn	Rubredoxin (C. Pasteur., Unconstr Ref)	1.2	R 3	3.79±0.13
5rxn	(Nrg + Xtal Ref)	1.2	R 3	3.81±0.04
2sga	Serine Proteinase (Strep. Griseus)	1.5	P 42	3.81±0.02
1sgc	(chymostatin complex)	1.8	P 42	3.80+0.03
3tln	Thermolysin (Native)	1.6	P 61 21 21	3.81±0.08
4tln	(L-Leu-NHOH)	2.3	P 61 21 21	3.81±0.08
5tln	(HONH-BZNMalonyl-A-G-Nitroanld)	2.3	P 61 21 21	3.81±0.08

Table 1.2 (cont'd)

PDB Code	Protein Description	Resolution (Å)	Space Group	(CA - CA)
1tpo	Trypsin, Bovine (Orthorhombic)	1.7	P 21 21 21	3.80±0.04
3ptn	(Trigonal, 2.4M (NH4)2SO4)	1.7	P 31 2 1	3.80±0.02
3ptb	(Benzamidine inhibited)	1.7	P 21 21 21	3.80±0.04
1tpp	/p-amidino-phenyl-pyruvate	1.4	P 21 21 21	3.80±0.05
3ptp	(DIP inhibited)	1.5	P 21 21 21	3.80±0.06
2ptc	Trypsin/Pancreatic Trypsin Inhibitor	1.9	I 2 2 2	3.78±0.04
1tpa	(anhydro trypsin)	1.9	I 2 2 2	3.79±0.05
4pti	Trypsin Inhibitor (Bovine)	1.5	P 21 21 21	3.79±0.04
5pti	(X-ray + Neutron)	1.8	P 21 21 21	3.80±0.04

Table 1.3

Correlation Tests to Resolutions

A. Correlation between Resolutions and Precisions of Structures Included in the Library

n = 79
slope = 0.002 ± 0.12
Correlation = 0.02

B. Correlations between Arithmetic Mean of the Resolutions of Structures Under Comparison and Intial SP rms, Final rms, and Percentage of dLDP Outliers

All comparisons in library

'Initial' fit	'Final'fit	dLDP analysis
n =56	n =56	n =56
slope = 0.37 ± 0.18	slope = 0.14 ± 0.07	slope = 3.8 ± 2.3
Correlation = 0.26	Correlation = 0.25	Correlation = 0.21

Lowest SP of group only

'Initial' fit	'Final'fit	dLDP analysis
n =25	n =25	n =25
slope = - 0.23 ± 0.21	slope = 0.01 ± 0.09	slope = - 0.9 ± 3.5
Correlation = - 0.26	Correlation = 0.25	Correlation = - 0.21

Highest SP of group only

'Initial' fit	'Final'fit	dLDP analysis
n =25	n =25	n =25
slope = -0.25 ± 0.22	slope = - 0.02 ± 0.09	slope = 0.9 ± 3.7
Correlation = - 0.24	Correlation = - 0.04	Correlation = 0.05

Table 1.4

Structural Comparisons of the Library

aa = number of amino acids
No. = number of outliers determined by SP of dLDP analysis
Individual = dLDP analysis mean & standard deviation for individual comparison

Comparison	aa	Superpositions Initial	Final	No.	dLDP Analysis Individual	No.
4atc-5atc	926	0.65	0.42	36	0.0±0.9	141
5cha-2cha	236	0.60	0.45	4	-0.1±1.1	43
-4cha1		0.42	0.15	20	0.0±0.8	9
-4cha2		0.46	0.19	21	0.0±0.8	8
-5cha2		0.49	0.22	17	0.0±0.9	12
2cna-3cna	237	1.00	0.84	10	0.1±1.3	71
1cpv-2cpv	108	0.22	0.22	0	0.1±0.5	0
3cyt-3cyt2	103	0.23	0.20	5	0.0±0.4	1
-4cyt		0.39	0.27	13	-0.1±0.4	3
2c2c-3c2c	112	0.11	0.09	2	0.0±0.2	0
1cts-2cts	437	2.40	0.57	125	0.0±0.8	51
-4cts1		2.37	0.67	108	0.0±1.1	103
-4cts2		2.39	0.69	102	0.0±1.1	102
3fxn-4fxn	138	0.29	0.25	5	0.1±0.5	3
2hhb-1hhoa*	287	0.94	0.71	21	0.2±0.7	26
-1hhob*		0.89	0.53	38	0.2±0.6	27
-2hcoa*		0.96	0.65	36	0.0±0.7	27
-2hcob*		0.92	0.54	36	0.0±0.7	30
2mhb-2dhb	287	0.95	0.71	18	-0.2±1.0	59
1hmq-1hmz	452	0.17	0.16	3	0.0±0.2	0
2pka1-2pka2	232	0.42	0.28	19	0.1±0.6	10
1lh1-1lh2	153	0.13	0.12	1	0.0±0.2	0
-1lh3		0.12	0.11	3	0.0±0.2	0
-1lh4		0.16	0.16	0	0.0±0.2	0
-1lh5		0.12	0.12	1	0.0±0.2	0
-1lh6		0.21	0.18	6	0.0±0.3	1
-1lh7		0.18	0.16	5	0.1±0.3	2

Table 1.4 (cont'd)

Comparison	aa	Superpositions Initial	Final	No.	dLDP Analysis Individual	No.
1lzt-2lyz	129	0.74	0.59	11	0.1±0.8	21
-7lyz		0.59	0.37	10	0.0±0.8	18
-1lym1		0.78	0.58	9	-0.1±1.0	34
-1lym2		0.87	0.65	11	0.0±1.2	37
1mbd-2mbn	153	0.40	0.24	8	0.0±0.4	1
-3mbn		0.38	0.21	9	0.0±0.4	2
-1mbo		0.53	0.09	2	0.1±0.3	1
-1mb5		0.54	0.29	10	0.1±0.5	7
1bp2-3bp2	123	0.48	0.31	4	0.0±0.6	4
9pap-1ppd	212	0.22	0.20	5	0.1±0.3	1
-1pad		0.54	0.49	8	0.0±0.8	32
1pcy-2pcy	99	0.12	0.12	4	0.0±0.1	0
-3pcy		0.17	0.14	3	0.0±0.2	0
-4pcy		0.12	0.11	3	0.0±0.2	0
-5pcy		0.12	0.11	2	0.0±0.2	0
-6pcy		0.16	0.13	7	0.0±0.2	0
1rn3-5rsa	124	0.19	0.15	7	0.0±0.3	0
-6rsa		0.24	0.21	5	0.0±0.4	1
-1rsm		0.48	0.40	7	-0.1±0.4	1
4rxn-5rxn	54	0.29	0.10	6	0.0±0.4	1
2sga-1sgc	181	0.11	0.10	5	0.0±0.2	0
3tln-4tln	316	0.15	0.15	0	0.0±0.3	0
-5tln		0.17	0.17	1	0.0±0.3	0
1tpo-3ptn	223	0.26	0.19	18	-0.1±0.3	1
-3ptb		0.11	0.11	0	0.0±0.2	0
-1tpp		0.11	0.11	0	0.0±0.2	0
-3ptp		0.21	0.14	12	0.0±0.3	1
2ptc-1tpa	281	0.12	0.11	7	0.0±0.2	0
4pti-5pti	58	1.24	0.36	4	-0.1±0.8	1

* a = first two subunits of hemoglobin (141 + 146 aa)
* b = second two subunits of hemoglobin (141 + 146 aa)

Table 1.5

dLDP, SP, and B Value Correlations to Center of Mass

Norm = (slope/Bmax)(1000)
x = no B values reported
- - - = B values all reported as 0
??? = some B values are not reported
suffixes after PDB entry denote subunit number(s) used in comparison
* = see Table 1.4 for description

	dLDP			**SP**			**B VALUES**							
Entries	Slope	SD	Corr	Slope	SD	Corr	Slope	Norm	SD	Corr	Slope	Norm	SD	Corr
4atc-5atc	.00	.00	-.02	.00	.00	.09	.05	*11*	.03	.06	.06	*12*	.03	.07
12	-.01	.01	-.13	.02	.00	.36	.32	*73*	.03	.40	.34	*65*	.04	.40
34	.00	.00	-.05	.01	.00	.28	.34	*77*	.03	.43	.36	*69*	.03	.44
1	.00	.01	-.03	.01	.00	.27	.48	*109*	.06	.41	.62	*119*	.07	.48
2	-.01	.02	-.06	.06	.01	.32	.39	*89*	.12	.27	.24	*46*	.16	.12
3	.00	.01	-.04	.00	.00	.13	.63	*143*	.06	.53	.61	*117*	.06	.51
4	.01	.01	.06	.01	.00	.17	.45	*102*	.10	.35	.52	*99*	.11	.36
5cha-2cha	.03	.02	.15	.02	.01	.17	.83	*218*	.05	.76	x	*x*	x	x
-4cha1	.01	.01	.05	.01	.01	.15					.59	*164*	.08	.46
-4cha2	.00	.01	.01	.02	.01	.23					.72	*206*	.06	.61
-5cha2	.01	.01	.03	.02	.01	.24					1.03	*271*	.06	.74
2cna-3cna	.01	.02	.04	.04	.01	.40	x	*x*	x	x	x	*x*	x	x
1cpv-2cpv	-.02	.02	-.10	.01	.00	.02	x	*x*	x	x	1.17	*89*	.58	.19
3cyt1-3cyt2	.03	.01	.21	.02	.01	.31	.62	*180*	.22	.27	.79	*168*	.23	.32
-4cyt	.01	.02	.03	.03	.01	.28					.44	*167*	.11	.31
2c2c-3c2c	.00	.01	.00	.00	.00	.14	1.00	*179*	.18	.47	1.00	*183*	.19	.46
1cts-2cts	-.01	.01	-.06	.13	.02	.36	.21		.04	.24	.31	*62*	.04	.37
-4cts1	-.01	.01	-.06	.13	.02	.37					.18	*36*	.07	.13
-4cts2	-.01	.01	-.06	.13	.02	.37					.19	*38*	.06	.14
3fxn-4fxn	-.01	.01	-.05	.02	.00	.41	x	*x*	x	x	x	*x*	x	x
2hhb-1hhoa	.00	.01	.02	.06	.01	.55	.89	*150*	.07	.58	1.23	*123*	.18	.37
-1hhob	.00	.01	.05	.05	.01	.48	.85	*143*	.08	.53				
1	.01	.19	.06	.01	.50	.94	1.29	*217*	.15	.47			.33	.42
2	.02	.01	.11	.02	.01	.23	1.28	*216*	.14	.60	1.08	*108*	.37	.24
tetramer	-.01	.00	-.13	.05	.01	.26	.81	*136*	.06	.52	---	---	---	---
-2hcoa*	.00	.01	.02	.06	.01	.55					x	*x*	x	x
-2hcob*	.00	.01	-.01	.05	.01	.41					x	*x*	x	x
1	.01	.01	.04	.06	.01	.45								
2	-.01	.01	-.03	.02	.01	.16								

Table 1.5 (cont'd)

	dLDP			SP			B VALUES							
Entries	Slope	SD	Corr	Slope	SD	Corr	Slope	Norm	SD	Corr	Slope	Norm	SD	Corr
2mhb-2dhba	.00	.01	-.03	.05	.01	.42	.01	*36*	.00	.20	x	*x*	x	x
1	.02	.02	.07	.07	.01	.49	.00	*0*	.00	.10	x	*x*	x	x
2	.01	.02	.02	.03	.01	.16	.02	*72*	.00	.29	x	*x*	x	x
1hmq-1hmz	.00	.00	-.03	.00	.00	.22	.23	*64*	.02	.42	.23	*61*	.03	.39
2pka1-2pka2	.01	.01	.09	.03	.00	.43	.84	*165*	.11	.46	.85	*174*	.11	.47
1lh1-1lh2	.00	.00	.12	.00	.00	-.03	1.41	*261*	.16	.57	1.41	*218*	.20	.50
-1lh3	.00	.00	.07	.00	.00	-.14					1.57	*243*	.21	.53
-1lh4	.01	.00	.12	.00	.00	.14					1.39	*257*	.19	.52
-1lh5	.00	.00	.09	.00	.00	.10					1.52	*242*	.20	.52
-1lh6	.01	.01	.13	.01	.00	.31					1.61	*241*	.21	.53
-1lh7	.01	.01	.11	.00	.00	.18					1.63	*259*	.22	.51
1lzt-2lyz	.02	.02	.08	.04	.01	.32	.29	*127*	.09	.27	x	*x*	x	x
-7lyz	.01	.02	.02	.03	.01	.26					x	*x*	x	x
-1lym1	.00	.02	.00	.04	.01	.33					.52	*217*	.08	.51
-1lym2	.02	.03	.07	.05	.01	.40					.46	*192*	.08	.46
1mbd-1mbn	-.02	.01	-.15	.03	.01	.29	.60		.12	.37	x	*x*	x	x
-2mbn	.00	.01	-.02	.02	.01	.27					.01	*56*	.00	.16
-3mbn	.00	.01	-.04	.02	.01	.27					.01	*40*	.00	.14
-1mbo	.01	.01	.22	.01	.01	.08					.49	*91*	.12	.33
-1mb5	.01	.01	.07	.02	.01	.23					x	*x*	x	x
1bp2-3bp2	.03	.01	.16	.01	.01	.13	.57	*98*	.07	.57	1.18	*236*	.16	.56
9pap-1ppd	.03	.01	.15	.01	.00	.44	.86	*150*	.08	.58	.63	*184*	.06	.61
-1pad	.02	.01	.14	.02	.00	.27					x	*x*	x	x
1pcy-2pcy	.06	.00	.16	.01	.00	.27	.34	*161*	.11	.29	.37	*175*	.10	.35
-3pcy	.01	.01	.11	.00	.00	.14					.54	*254*	.10	.49
-4pcy	.01	.01	.15	.01	.00	.33					.57	*197*	.17	.32
-5pcy	.01	.00	.30	.01	.00	.48					.43	*149*	.13	.31
-6pcy	.01	.01	.19	.01	.00	.24					.62	*203*	.15	.39
1rn3-5rsa	.00	.01	.05	.01	.00	.23	.01	*250*	.00	.31	.63	*273*	.05	.72
-6rsa	.00	.01	-.03	.01	.00	.15					.69	*286*	.05	.78
-1rsm	-.01	.01	-.14	.03	.01	.41					.93	*296*	.10	.63
4rxn-5rxn	-.01	.03	-.07	.06	.01	.56	3.14	*???*	.59	.59	1.70	*109*	.51	.42
2sga-1sgc	.00	.00	.09	.00	.00	.23	.77	*182*	.08	.57	.77	*212*	.08	.57
3tln-4tln	.00	.00	.04	.00	.00	.03	.36	*104*	.04	.48	.33	*126*	.03	.48
-5tln	.00	.00	.06	.00	.00	.07					.32	*128*	.03	.52

Table 1.5 (cont'd)

	dLDP			SP			B VALUES							
Entries	Slope	SD	Corr	Slope	SD	Corr	Slope	Norm	SD	Corr	Slope	Norm	SD	Corr
1tpo-3ptn	.00	.00	-.02	.02	.00	.45	.53	*77*	.05	.61	.72	*166*	.08	.53
-3ptb	.00	.00	-.05	.00	.00	.16					.61	*196*	.05	.61
-1tpp	-.01	.00	-.12	.00	.00	.14					.70	*167*	.08	.49
-3ptp	.01	.00	.01	.01	.00	.29					.56	*140*	.06	.51
2ptc-1tpa	.00	.00	.04	.00	.00	.20	.44	*94*	.06	.41	.41	*106*	.05	.43
4pti-5pti	-.01	.03	-.05	.13	.04	.39	.87	*193*	.20	.50	1.09	*236*	.21	.57

Table 1.6

B Value Means and Ranges of the Library

d = radius of protein (from center of mass to furthest alpa carbon)
Ratio = ration of highest to lowest reported B value
- = no B values reported or all reported as 0

		Alpha carbons			Side Chains			All atoms
Entry	**d**	**Mean±sd**	**Range**	***Ratio***	**Mean±sd**	**Range**	***Ratio***	**Mean±sd**
4atc	56.2	17.8±7.9	3.0-44.0	*15*	20.7±4.4	3.0-58.3	*19*	19.8±8.6
5atc		17.5±8.4	3.0-52.1	*17*	20.4±4.4	3.0-53.8	*18*	19.5±8.9
5cha1	25.6	15.2±5.0	5.5-38.0	*7*	16.5±3.7	5.7-35.1	*6*	15.7±5.5
2cha		0	0	*0*	0	0	*0*	0
4cha1		13.7±5.9	5.2-36.0	*7*	14.6±3.7	4.4-36.4	*8*	14.0±6.0
4cha2		13.5±5.4	5.0-34.9	*7*	14.2±3.3	5.1-35.3	*7*	13.9±5.5
5cha2		15.4±6.4	6.2-38.0	*6*	17.0±4.4	4.9-38.2	*8*	16.1±6.7
2cna	33.5	-	-	-	-	-	-	-
3cna		-	-	-	-	-	-	-
1cpv	18.5	9.0±0.0	9.0-9.0	*1*	9.0±0.0	9.0-9.0	*1*	9.0±0.0
2cpv		17.9±22.8	2.0-131.6	*66*	20.2±13.4	2.0-144.3	*72*	18.3±20.8
3cyt1	17.0	18.3±5.7	5.6-33.3	*6*	26.2±6.8	8.5-68.0	*8*	22.8±9.9
3cyt2		17.2±6.0	1.1-46.9	*43*	25.3±7.2	4.0-81.2	*20*	21.8±10.3
4cyt		15.1±3.6	8.4-26.3	*3*	22.1±6.0	6.9-96.3	*14*	18.6±9.8
2c2c	20.4	12.1±6.5	3.5-56.0	*16*	16.6±5.3	2.6-66.7	*26*	15.0±7.7
3c2c		9.5±6.7	1.8-54.7	*30*	13.7±5.5	2.0-64.5	*32*	12.2±7.9
1cts	44.0	23.2±6.7	6.7-42.5	*6*	25.0±4.6	6.0-50.0	*8*	24.2±7.5
2cts		14.4±6.1	5.0-50.0	*10*	16.2±4.4	5.0-50.0	*8*	15.4±7.2
4cts1		21.6±9.9	6.0-50.0	*8*	21.6±5.5	6.0-50.0	*8*	21.7±10.3
4cts2		20.6±9.5	6.0-50.0	*8*	21.4±4.9	6.0-50.0	*8*	21.4±9.1
3fxn	20.2	-	-	-	-	-	-	-
4fxn		-	-	-	-	-	-	-
2hhb	33.7	21.0±8.8	6.1-59.4	*10*	27.8±6.6	5.8-80.6	*14*	24.3±11.4
1hho		35.2±18.4	5.0-100.0	*20*	44.3±11.0	5.0-100.0	*20*	39.1±20.4
2hco		-	-	-	-	-	-	-
2mhb	28.1	1.5±0.2	1.1-2.8	*3*	1.6±0.2	1.1-2.8	*3*	1.5+0.3
2dhb		-	-	-	-	-	-	-
1hmq	51.1	15.1±5.7	4.9-36.1	*7*	16.0±3.6	3.1-36.4	*12*	18.7±13.1
1hmz		14.9±6.1	4.5-37.6	*8*	16.0±3.7	1.8-37.2	*21*	19.1±15.5

Table 1.6 (cont'd)

Entry	d	Alpha carbons Mean±sd	Alpha carbons Range	Alpha carbons *Ratio*	Side Chains Mean±sd	Side Chains Range	Side Chains *Ratio*	All atoms Mean±sd
2pka1	24.3	15.1±8.5	5.5-50.8	*9*	17.9±5.6	5.0-55.8	*11*	16.6±9.7
2pka2		19.5±8.8	5.9-48.9	*8*	22.8±6.0	5.0-61.3	*12*	21.2±10.4
1lh1	24.7	15.8±10.6	3.0-54.0	*18*	21.1±6.7	3.0-56.2	*19*	19.0±11.2
1lh2		17.5±12.1	3.0-64.6	*22*	22.2±7.5	3.0-64.0	*21*	20.3±12.8
1lh3		17.5±12.8	3.0-64.6	*22*	22.9±7.5	3.0-68.8	*23*	20.9±13.2
1lh4		17.0±11.6	3.0-54.0	*18*	22.2±7.2	3.0-61.3	*20*	20.5±12.1
1lh5		17.4±12.6	3.0-62.7	*21*	22.4±7.8	3.0-68.8	*23*	20.4±13.2
1lh6		16.3±13.1	3.0-66.9	*22*	22.9±7.9	3.0-68.5	*23*	20.7±13.5
1lh7		17.7±13.8	3.0-63.0	*21*	23.6±8.3	3.0-69.3	*23*	21.7±14.1
1lzt	22.0	9.3±4.4	4.0-22.8	*6*	10.8±2.6	4.0-27.0	*7*	10.1±4.7
2lyz		1.5±0.0	1.5-1.5	*1*	1.5±0.0	1.5-1.5	*1*	1.5±0.0
7lyz		-	-	-	-	-	-	-
1lym1		13.8±4.2	4.0-24.0	*6*	14.9±2.7	2.0-34.0	*17*	14.4±5.0
1lym2		12.7±4.0	2.0-24.0	*12*	13.9±3.0	1.0-31.0	*31*	13.3±4.9
1mbd	22.7	10.5±6.7	4.4-59.1	*13*	18.7±5.9	4.7-65.1	*14*	14.6±9.9
2mbn		1.4±0.1	1.1-1.8	*2*	1.5±0.1	1.1-2.5	*2*	1.5±0.2
3mbn		1.4±0.2	1.1-2.5	*2*	1.5±0.1	1.1-2.5	*2*	1.5±0.2
1mbo		11.3±6.1	4.0-53.9	*14*	18.9±5.2	4.0-55.9	*14*	15.3±9.0
1mb5		1.5±0.1	1.3-1.7	*1*	1.5±0.0	1.3-1.8	*1*	1.5±0.1
1bp2	23.1	6.9±4.3	0.7-19.1	*27*	15.1±5.0	1.0-59.4	*59*	11.6±10.0
2bp2		9.0±11.5	1.0-50.0	*50*	16.5±7.9	1.0-50.0	*50*	13.7±14.4
9pap	26.4	11.8±7.0	1.0-57.3	*57*	19.7±6.1	1.0-74.4	*74*	15.9±12.2
1ppd		12.3±4.9	4.8-34.3	*7*	18.2±5.3	2.4-86.4	*36*	15.3±10.5
1pad		-	-	-	-	-	-	-
1pcy	19.2	9.5±3.9	2.0-21.1	*11*	18.1±6.5	2.0-81.0	*41*	14.0±11.1
2pcy		8.0±3.5	2.0-21.1	*11*	17.2±6.4	2.0-89.4	*45*	13.0±11.7
3pcy		12.0±3.6	6.0-21.3	*4*	13.0±2.2	5.3-27.9	*5*	12.4±4.4
4pcy		10.9±5.9	2.0-29.0	*15*	19.6±8.3	2.0-96.2	*48*	15.8±13.4
5pcy		10.5±4.5	2.0-28.9	*15*	19.8±6.8	2.0-85.0	*43*	15.5±12.2
6pcy		10.8±5.2	2.0-30.6	*15*	20.23±7.2	2.0-93.7	*47*	15.68±13.2
1rn3	22.7	0.2±0.1	0.1-0.4	*4*	0.3±0.1	0.0-0.9	*x*	0.2±0.2
5rsa		10.6±3.7	4.0-23.1	*6*	12.5±2.2	3.7-32.6	*9*	11.9±5.5
6rsa		13.4±3.8	5.0-24.1	*5*	14.7±1.8	3.9-34.9	*9*	14.4±5.5
1rsm		11.4±6.2	4.5-31.4	*7*	13.6±3.8	2.7-38.2	*14*	12.4±7.1
4rxn	16.0	-	-	-	-	-	-	-
5rxn		17.4±23.8	7.5-156.2	*21*	30.5±24.1	7.4-252.5	*34*	23.2±30.7

Table 1.6 (cont'd)

		Alpha carbons			Side Chains			All atoms
Entry	**d**	**Mean±sd**	**Range**	***Ratio***	**Mean±sd**	**Range**	***Ratio***	**Mean±sd**
2sga	22.1	12.4±5.6	5.2-42.4	*8*	15.7±4.1	5.7-56.0	*10*	14.0±7.0
1sgc		11.0±5.7	2.5-36.3	*15*	13.6±4.6	2.0-54.8	*27*	12.4±7.1
3tln	34.4	13.8±4.6	6.8-34.5	*5*	15.5±2.9	6.5-39.7	*6*	14.6±5.4
4tln		9.6±4.3	1.9-26.3	*14*	10.7±2.8	0.8-37.0	*46*	10.4±5.2
5tln		9.9±3.9	1.9-25.0	*13*	11.0±2.7	0.6-35.1	*59*	10.6±4.9
1tpo	23.8	16.2±4.0	8.9-30.7	*3*	16.9±2.4	6.3-40.3	*6*	16.5±4.5
3ptn		14.5±6.3	4.6-43.4	*9*	15.5±4.5	1.4-50.7	*36*	14.9±6.9
3ptb		13.0±4.6	4.1-31.1	*8*	14.0±2.9	3.1-41.4	*13*	13.5±5.0
1tpp		9.2±6.6	0.1-42.0	*420*	11.2±4.0	0.1-49.4	*494*	10.8±7.1
3ptp		9.9±5.1	4.0-40.0	*10*	15.4±3.6	4.0-40.0	*10*	13.0±7.7
2ptc	34.7	24.1±6.6	10.2-46.6	*5*	26.1±4.9	6.7-55.6	*8*	25.1±7.5
1tpa		18.7±5.9	5.9-38.8	*7*	20.4±4.1	5.0-46.7	*9*	19.5±6.5
4pti	18.1	11.4±6.1	4.1-45.1	*11*	16.6±4.0	4.3-50.1	*12*	14.6±7.8
5pti		13.9±6.8	6.8-46.1	*7*	17.0±4.3	5.8-37.1	*6*	15.9±7.2

Chapter 2

Trypsin Crystal Structures Analyses

Goal

To compare and contrast the conformational variabilities, B values, solvent accessibilities, and hydrogen bonding patterns of the seven β-trypsin crystal structures included in the library of Chapter 1, with focus on trypsin's three physiological sites.

2.1 Evaluation of Crystallization Media - Was Calcium Present in All Cases?

All seven trypsin PDB structures included in the library of Chapter 1 have a calcium ion coordinate included by the crystallographers. A potential important inconsistency, however, exists in the 3ptp, 2ptc, and 1tpa x-ray diffraction studies. In the DFP-inhibited (Chambers & Stroud, 1979), native trypsin-PTI (Ruhlmann et al., 1973), and anhydro trypsin-PTI (Marquart et al., 1983) experiments respectively, no calcium was reported to be introduced in the crystallization medium. Arguments presented below support the contention that these structures therefore, most probably, do not have a calcium bound.

Stroud [personal communication] said that the investigators made no attempt to remove calcium, bound at the "high affinity site", with calcium chelators such as EDTA. He theorized that calcium bound from the initial purifications was not removed in the subsequent dialysis. Atomic absorption of dried samples of dialyzed trypsin that were used in the FTIR experiments of Chapter 4 indicated that less that 0.5 ppm of residual calcium remained in the dried powder [M. Thompson, personal communications].

Interestingly, there have been experiments performed on trypsin which utilized dialysis against a solution containing no calcium to completely remove bound calcium. The experiments of DeJersey et al. (1980) found an enhancement of terbium luminescence upon its binding to the calcium site. Before the studies "Bovine and porcine trypsin and alpha-chymotrypsin were dissolved in and exhaustively dialyzed against 1 mM HCl at 4°C to remove metal ions." There is no mention of a test of the calcium content after the dialysis.

Another report (Gabel & Kasche, 1973), which studied the influence of calcium and heat on trypsin autolysis, supports the contention that the calcium is not bound strongly enough to be present after dialysis. They concluded by $^{45}Ca^{2+}$ binding to β-trypsin and subsequent gel filtration that "The complex of beta-trypsin and calcium therefore has a life time considerably shorter than one hour, the time needed for the experiment [Sephadex gel filtration]".

Moreover, some inconsistent comments in published studies by Stroud and coworkers also support this claim. Chambers & Stroud (1976) describe, in part, the crystallization of DIP-trypsin:

> "Refinement of the structure from residue Asn 72 to Gln 81 indicated a tightly bound positive ion and second well-ordered solvent molecules, which were previously not resolved in the 2.7Å MIR map. Bode and Schwager (1975a,b) in their independent refinement of the structure of benzamidine-inhibited bovine trypsin recognized this area as the primary calcium ion binding site. While the refined structure of DIP-trypsin arrived at from the present study is nearly identical in this area to that reported by Bode and Schwager, the density for the positive ion in the present study is about half of that expected for a fully occupied calcium ion. It is possible that this site is partially occupied by Ca^{2+}; however, the ligands do not appear disordered as expected for such a situation. Since the crystallizing solution contained a high concentration of Mg^{2+} (5.8% $MgSO_4$), occupancy by a magnesium ion is another possibility in spite of the difference in atomic radius between Ca^{2+} and Mg^{2+}. Occupancy by a water molecule is not as likely, because of the high density of negative charge surrounding this site." (Chambers & Stroud, 1977).

In the work describing the crystallization of trypsinogen (Kosiakoff et al., 1977) they state:

> " . . . In our case, no Ca^{2+} was added to any of the crystallizing solutions and no attempt has been made to determine the Ca^{2+} content in the crystals.
>
> The ion found at the primary site in DIPT [DIP-trypsin] is probably a magnesium ion present in the crystallizing liquor (5.8% $MgSO_4$). The electron density at this site refined to that expected for a water molecule (10 electrons) or a magnesium ion (Chambers and Stroud, 1976). Since Ca^{2+} accelerates autoactivation of trypsinogen to trypsin, reasonable care was taken to exclude it from the trypsinogen-crystallizing solution. Ca^{2+} was present in the buffer (0.02M in $CaCl_2$) used during the chromatographic purification,

> although the solution was later dialyzed using large volumes of 10^{-3} M HCl. Thus, it was surprising to find that the bound ion at the primary ion site in Tg [trypsinogen] refined to an occupancy of 18 electrons. We presume this to be a single site, fully occupied by a single, tightly bound, Ca^{2+} ion, which remained as an integral component of the zymogen throughout."

Since the affinity of calcium for the "high affinity" site (K_d = 0.1 mM) is the same for both trypsin and trypsinogen, why does trypsinogen have a fully occupied calcium bound while DIP-trypsin has a magnesium ion [most likely] bound? Although these facts do not prove that calcium was not present in the three crystals, they lend credible support to the contention that none was bound during crystallization and x-ray data collection.

A significant point of dispute arises, however, if one assumes that these three trypsin crystal structures have no calcium bound and that the solution conformation of the enzyme is similar to that in the crystal state. Since all three of these PDB entries also have an active site inhibitor bound (either covalent or macromolecular) and are shown below to have their autolysis sites perturbed, one can ask - is the cause of this perturbation the lack of calcium ion bound or the active site inhibition? In other words, are these perturbations due to the inhibitors propagating a change through residues which comprise the activation domain of trypsin, or are they the changes hypothesized to occur to this site in protecting trypsin from autolysis?

If the perturbation is due to the lack of calcium, with no contribution from the active site inhibition, this would, in itself, be indicative of the type of change induced by calcium. However, the FTIR experiments presented in Chapter 4 indicate that it is probably the inhibitors that perturb the autolysis site conformation because calcium has no detectable effect on the secondary structure of inhibited trypsins. This possibility, and its significance, are assessed in the specific trypsin analyses presented below, in modeling of the autolysis dimer (Chapter 3), and in evaluation of the mechanism of action of calcium in slowing trypsin autolysis (Chapter 6).

2.2 Results

2.2.1 PTI Contacts

Table 2.1 lists the vdW contacts between trypsin and pancreatic trypsin inhibitor in the 2ptc crystal structure. These contacts are also included in Appendices A, B, and C to facilitate analyses of these data. 30 residues of trypsin make contacts within PTI. Of these, 17 are in contact with only the P5-P1 and 24 are only in contact with the P1'-P5' residues of PTI's reactive site. The remainder are in contact with residues 34V, 36G, 37G, 38C, and 39R of PTI. Residue 192Q of trypsin has the most contacts to PTI, with extensive backbone and sidechain interactions. 41F, 193G, and 215W also have large numbers of backbone contacts and have similar solvent accessibilities of 15 - 20%. Two tyrosine residues, 39Y and 151Y, are unique in that they have extensive sidechain contacts but few backbone interactions. Both are also exposed about 50% to solvent.

15 of PTI's 58 residues are in contact with trypsin. Of these, the P3-P3' residues of the reactive site and 36G-37G-38C make extensive backbone interactions with trypsin. The interaction of the reactive site P3-P3' residues is shown to be symmetric, with about the same degree of interaction on the amino terminus side of the reactive site P1 bond as on the carboxy terminus side. Residue 39R is found to have predominantly sidechain contacts.

2.2.2 Alpha Carbon B Values of the Three Physiological Sites of Trypsin

Analyses of the alpha carbon B values of the active, calcium, and autolysis sites of the seven crystal structures are shown in Table 2.2. 'Full' denotes the average and standard deviation of the entire 223 alpha of the enzyme. Numbers in parentheses after the structure identification are the number of alpha carbons which had thermal mobility too great to determine. 'Range' is the range of the minimum to maximum alpha carbon B values for the entire enzyme.

'Active' are the mean and standard deviations of the B values of the 14 residues comprising the active site (see Methods). 'Calcium loop' refers to the 11 residues (70E -

80E) which comprise the calcium binding site according to Huber (Huber, 1978). 'Autolysis' denotes the ten residues which comprise the P5-P5' residues of the 145K autolysis site of β-trypsin.

The following facts are gleaned from observations in Table 2.2, along with Appendices B and C:

Active Site

1.a. In the five uncomplexed trypsin structures the 14 residues which comprise the active site have the lowest B values of the three sites examined. They also show about 20% less thermal variability than the entire enzyme average.

b. The same results are found in active sites of the two complexed structures.

Calcium Binding Loop

2.a. The B values of the calcium loop are found to be intermediate between those of the active and autolysis sites. However, all five uncomplexed structures do not have thermal mobilities less than that of the entire enzyme. Two structures, 1tpo and 3ptn, have calcium loop average B values greater than the entire enzyme average. The other three structures (3ptb, 1tpp, and 3ptp) are below that average.

b. The calcium loops of the two complexed structures do not show the same trends as the uncomplexed structures. In these two structures the thermal mobilities are above the active site, but are about the same as that of the autolysis site. Both are above the entire enzyme average.

Autolysis Site

3.a. The individual and average B values of the autolysis site are the highest of the three areas examined and are significantly higher than the average B value of the entire enzyme. Moreover, it is apparent that the thermal mobilities of the P1'-P5' residues are always much higher than the P5-P1 residues and are on the order of 1.6 to 3.7 times higher than the active site.

b. In the two complexed trypsin structures the same thermal mobilities are found except that the average of the entire autolysis site is not higher than the 2ptc calcium loop average.

2.2.3 Hydrogen Bonding Patterns of the Calcium and Autolysis Loops

Table 2.3 lists the hydrogen bonding patterns of the calcium and autolysis loops. The seven structures have similar hydrogen bonding patterns with some variability in the calcium binding loops. Five hydrogen bonds are formed within the autolysis loop in all seven crystal structures. One identical hydrogen bond (141NE1-71O) is found between the calcium binding and autolysis loops in all structures. 143N of the autolysis loop is also hydrogen bonded to 192Q (a residue comprising the active site) in all seven structures. Finally, the N terminus (16I) is hydrogen bonded to the P3 residue of the autolysis loop (143N) in all seven structures.

2.2.4 Trypsin Crystal Contacts

Of the seven trypsin crystal structures included in the library of Chapter 1, six crystallize to space group P 21 21 21. Only one, 3ptn, crystallizes to a different space group - P 31 2 1. In Appendix A are listed the crystal contacts in the 1tpo (chosen to represent the six structures which crystallize to space group P 21 21 21) and 3ptn unit cells. The 38G, 86S, 87K, 88S, 90V, 92P, 202S, and 203G residues of β-trypsin are shown to make extensive contacts in both crystals. Active site 195S makes no contacts in either.

Below is a synopsis of the total number of residues which make either backbone or sidechain contacts, the number of residues which make backbone contacts, the number of residues which make backbone-backbone contacts, and the number of each type in common between the two unit cell representatives in the entire enzyme and the three physiological sites of trypsin:

Entire enzyme

	1tpo	3ptn	Common
Total	66	79	53
Backbone	49	56	34
Backbone-Backbone	23	30	14

Active Site

	1tpo	3ptn	Common
Total	7	9	7
Backbone	7	5	5
Backbone-Backbone	1	2	0

Autolysis Site

	1tpo	3ptn	Common
Total	5	2	2
Backbone	5	2	2
Backbone-Backbone	3	2	2

Calcium Binding Loop

	1tpo	3ptn	Common
Total	4	4	4
Backbone	2	2	2
Backbone-Backbone	1	1	1

2.2.5 Trypsin Superpositions

Figure 2.1 depicts the 1tpo crystal structure with residues defined in the Methods section as constituting trypsin's active site highlighted with van der Waals surfaces. Residue 195S is individually labeled. Similarly, Figure 2.2 depicts with van der Waals surfaces the spatial and topographical relationships between the calcium binding loop and the P5-P5' residues of the β- and α-trypsin autolysis sites. Residue 195S is again individually labeled. Figure 2.3 depicts the relationships between the 1tpo calcium binding loop, the P1 residues of the β- (145K) and α-trypsin (188K) autolysis sites, 141W, 195S, and the N-terminus (16I) by generation of their respective van der Waals surfaces.

In Appendix B are listed the results of the SP comparisons between the four uncomplexed and two complexed trypsins with the 1tpo crystal structure. Several individual and contiguous residues are identified as outliers by the SP method:

1. The calcium binding loop of 3ptn is disturbed, with 76V, 77E, and 78G, being determined as outliers. The 3ptp and 2ptc structures have 78G determined as an outlier, while the 1tpa structure has 78G and 79N determined as outliers.

2. The autolysis site conformation of the 3ptp structure is found to be different than the 1tpo structure. Six of the its residues are identified as outliers. In the two complexed structures, the P3'-P5' residues are determined to be outliers.

3. Very few residues which comprise the active site are identified as outliers by the SP method in the six comparisons. The 1tpa structure has 97N and 98T identified, with deviations around 0.7 Å. The DIP-inhibited structure (3ptp) has only 219G identified as an outlier.

4. Residues 115N, 116S, and 117R are shown to be variable in the 3ptn and two complexed structures.

5. Several contiguous alpha carbon positions in the 3ptn structure are found to vary from the 1tpo structure. These are: 165D-166S-167S; 171A-172Y-173P-174G; and 202S-203G. These residues have deviations of 0.46 to 0.83 Å.

6. Residue 153D (9 Å from the calcium ion) is found to be an outlier in the two complexed structures, with relatively large deviations of 0.90 and 0.77 Å.

2.2.6 Solvent Accessibilities

The solvent accessibilities of the 223 amino acids of the seven trypsin crystal structures are tabulated in Appendix C. They are shown to vary only slightly amongst the five uncomplexed and two complexed trypsin structures (when PTI is removed for the calculation). 174G is found to be the most exposed residue in all seven trypsin structures. The P1'-P5' residues of the autolysis site exhibit the most variability of the three physiological sites. 145K is about 25% less exposed and 147S is about 25% more exposed in the 3ptp structure than in the 1tpo structure. In the 1tpp structure, 148G is 50% less exposed than in the 1tpo structure. Of the 14 active site residues, only 219G shows any appreciable change in accessibility upon complexation with PTI. None of the 11 residues which comprise the calcium binding site show any appreciable variability in solvent accessibility.

2.2.7 Thermal Variabilities

B Values of the seven trypsin structures are tabulated in Appendix D. Regions of the protein, other than the physiological sites, that have relatively high thermal mobility (as indicated by having values at least one standard deviation greater than the average) are:

61S-62G; 115N-116S-117R; 174G; and 184Y-185L-186E. All of these regions were also shown in Appendix B to have high solvent accessibilities. Residue 115N is found to have the highest alpha carbon B value in three of the seven structures (1tpo, 3ptb, and 1tpp), while residue 148G is found to have the highest alpha carbon B value in three others (3ptp, 2ptc, and 1tpa). The highest alpha carbon B value of the 3ptn structure is 78G, a residue that is part of the calcium binding loop.

Residues with relatively low alpha carbon B values are: 51W-52V-53S-54S-55A-56A; 103I-104M; 139S-140G; 196G-197G; 213V-214S; and 226G-227V-228Y-229T. Of these, only residue 213V comprises the active site. Other regions are contiguous to active site residues and have contacts to PTI but did not meet all of the criteria to be considered as part of the active site (see Methods).

Residue 63I is unique in that it is buried but has a relatively high thermal mobility in the uncomplexed structures. In the two complexed structures, its thermal mobility is found to fall below the average of the entire enzyme. Finally, the B values of residues 151Y and 153G are found to increase in the two complexed structures relative to the uncomplexed structures.

2.3 Discussion

Specific analyses of the seven trypsin structures included in the library of Chapter 1 were performed to compare and contrast their conformational response to different inhibitors and chemistry. Focus on the active, calcium binding, and autolysis sites was performed in an attempt to determine their relative variabilities and responses to different environment, chemistry, and physiological stimuli. The active site is particularly well-conserved, as shown by its small conformational and thermal variability, with the calcium binding and autolysis sites exhibit progressively greater variabilities.

The preservation of the active site conformation is consistent with the observations that macromolecular inhibitors adapt to the enzyme (Ruhlmann et al., 1973; Bode et al.,

1976; Bode & Schwager, 1975b). This is corroborated by the significant backbone conformational differences noted between free and complexed PTI in Chapter 1. The preservation of the active site has been hypothesized to result partly from an amino terminus - 194D salt bridge which acts as a "clamp" (Huber & Bode, 1978) and is shown to form a hydrogen bond in Appendix B. It has also been hypothesized to control the conformational change which occurs in trypsinogen activation (Stroud et al., 1977). Neither of these residues was identified as an outlier in any of the SP comparisons of trypsin structures. Both residues have lower than average alpha carbon B values in all cases except the 16I residue in the 3ptn structure.

This preservation of the conformation (and diminished thermal variability) of the active site is used as a basis for the modeling of the autolysis dimer in Chapter 3. One of the assumptions in that work is that the active site residues move no more than the extent found in any of the six crystal structure comparisons.

Residues 214S and 216G were first found by Stroud et al. (1971) to make interactions with the macromolecular inhibitors. Residue 192Q, shown to interact extensively with PTI in both inhibited structures, was identified as being a "polar flap covering the entrance to the binding pocket" (Krieger et al., 1974) and rearranging "completely" upon complexation (Bode et al., 1976). Here, it was determined to have lower than average B values and its alpha carbon position did not significantly change. This supports the contention that only its sidechain acts as the "polar flap". Residue 99L was also found by Bode et al. (1976) to rotate 180° about its CB-CD bond. The SP results indicate that its alpha carbon position also does not vary.

A possible important cause of the high thermal variability of the autolysis loop of the seven trypsin crystal structures is contamination of the crystals with α-trypsin. Stroud et al. (1971) estimated their trypsin crystals to be comprised of 50% β- and 50% α-trypsin, Bode and Schwager (1975b) estimated theirs to be 20% α-trypsin, Walter and Bode (1983) estimated theirs to be 90% β-trypsin, and Huber et al. (1974) estimated theirs to be 95% β-

trypsin. The SP-Sephadex column run to separate the two forms for FTIR experiments (see Chapter 4) showed that about 1/3 of the Sigma trypsin is α trypsin. The scission of the 145K-146S peptide bond would create a new N and C-terminus and would, very likely, increase the thermal variability of this region.

Limitations of the dLDP method, as outlined in this study, are shown here. The conservative application of the dLDP method is exemplified by the 1tpo-2ptc comparison. Complexation with a macromolecule would, at first, be thought of as inducing 'significant' changes in both trypsin and the inhibitor. However, the dLDP method of Chapter 1 identified only 3 outliers (1.3%). Either complexation does not induce these changes, or this is a consequence of the conservative use of 1.63 standard deviations of the library as criteria for exclusion.

More substantive and supportive evidence of the contention that complexation brings about 'significant' changes in the interacting molecules is seen in a previous study of trypsin (Liebman, 1986). The large family of trypsin structures is unique and one of the most extensive of the PDB. What may be important in comparisons in this family is the fact that the well-defined structures behave consistently across the family and small changes induced by environment and inhibitors, which are above baseline changes but within the Chi-square distribution, are significant.

Other support for the assertion that the conformational responses of trypsin to macromolecules are significant come from the work of Marquart et al. (1983). They feel that the orthogonal-trigonal trypsin comparison ". . . provides an objective upper limit of the mean error in alpha carbon positions." They note that the difference in the two structures results from ". . . both errors and true structural differences caused by lattice packing, solvent environment and/or different functional states . . ." and feel that the smallest values [here around 0.1 Å] may be close to the true error." An error of 0.1 Å in the coordinates would mean that the SP deviations in the complexed structure comparisons of 0.34 Å and greater are significant.

The pattern of residues involved in crystal contacts in the two trypsin space groups are similar in many respects. The extent of the contacts indicates that crystallization to different space grouping has little effect on the conformation of β-trypsin. The P 31 2 1 space grouping results in more total contacts. It has more active site residues in contact, fewer involving the autolysis site, and almost identical contacts involving the calcium binding loop. The SP results in Appendix B identify 19 alpha carbons as outliers, although the structures are, in general, very similar. Residues 115 - 117 exhibit the most conformational variability which could be due, in part, to a different pattern of crystal contacts.

The active site residues show similar contact patterns although 219G is much more involved in the 1tpo unit cell. None of these residues were identified as outliers by the SP method. The autolysis site of the 1tpo structure is much more extensively involved with other molecules of the unit cell but, again, shows little conformational variability from that of 3ptn. The similar autolysis site conformations of 1tpo and 3ptn supports the contention that the change in the 3ptp autolysis site is due to the inhibitor binding to the active site.

The hydrogen bonding pattern of the three physiological sites of trypsin reveals several patterns which are probably integral to the enzyme's structure and function. First, the sparse and weak hydrogen bonding pattern between the calcium binding site and the remainder of the entire protein (specifically the autolysis and active sites) supports the hypothesis that no signal is propagated to the remainder of the protein with calcium binding. This is consistent with the nmr studies of Chaincone et al. (1985) in which they found that ligand binding to the activation domain has no effect on the calcium loop and the terbium studies of DeJersey et al. (1980) in which they found that benzamidine binding to the active site has no effect on it's emission. By analogy, calcium binding would have no effect on the autolysis site. This argument is strengthened if one considers the 3ptp, 2ptc, and 1tpa structures to have no calcium bound. There are no consistent differences in the

hydrogen bonding pattern between the calcium loop and remainder of the protein, or of the solvent accessibilities of the calcium loop residues in these three structures.

Second, three hydrogen bonds are found between the autolysis loop and the activation domain of the enzyme. This explains how active site inhibition can propagate changes to the autolysis site, as shown in the 3ptp, 2ptc, and 1tpa studies. The autolysis and calcium loops, being of approximately the same number of residues, exhibit some similar patterns. A hydrogen bond exists between the side chain of 141W and the backbone of 71D. Both have about 10 -12 hydrogen bonds maximum with about one third of these existing in all seven trypsin crystal structures.

Similar features of the physiological response of the enzyme to the conditions tested are found in the report of Bode and Schwager (1975b). In their report, they identified nine residues as forming the active site, 12 residues as forming the calcium binding loop, and 38 residues as being in contact with buried water molecules. The comparisons with their site definitions indicate:

1. The active site conformation is preserved. One residue of it is identified as an outlier by the SP method in the 1tpo-3ptp comparison only, and its SP deviation is small. Furthermore, the average of the alpha carbon B values which comprise the active site is 14.2 ± 1.5, which is not significantly different than the average B value of the entire protein (16.2 ± 4.0).

2. The calcium binding loop has one Gly residue identified as an outlier by the SP method in only the 1tpo-3ptp and 1tpo-2ptc comparisons. The average of the alpha carbon B values of this site is 19.0 ± 5.5, slightly higher than the average of all 223 alpha carbons. Furthermore, the Gly residue that is identified as an outlier in the 1tpo-3ptp comparison also has the largest B value (27.1) of the 12 residues of the calcium binding site. The fact that this region shows more thermal variability than the entire structure, but smaller backbone structural change, is in opposition to the general correlation between thermal and conformational variabilities found in Chapter 1. This implies that either the maintenance of

the integrity of this region either has important physiological significance or it is an artifact of the refinement process.

3. Of the 38 residues hydrogen bonded to buried waters, only one is identified as an outlier by the SP method in both of the trypsin comparisons. The average of the 38 alpha carbon B values of these residues is 15.0±3.5, which is not significantly different from the average of the entire structure. Only 99R (B=24.1), identified as an outlier in the 1tpo-2ptc comparison, has a B value significantly higher than the average of these 38 residues.

Conclusion: Conformational changes, B value variabilities, and solvent accessibilities indicate that the active site of trypsin is conformationally conserved under a variety of conditions. The calcium binding site exhibits more variability, while the 145K autolysis site exhibits the largest variability, which is probably related to the binding of inhibitors to the active site. Being the result of evolutionary forces, these attributes are indicative of the physiological and chemical roles of these sites. They suggest that the active site conformation of the 'enzyme', essential for its activity against a wide variety of proteins, is conserved. The [autolysis loop of the] 'substrate' must, therefore, adapt to the active site during autolysis. The calcium loop is entirely exposed to solvent and possesses few hydrogen bonds to the remainder of the enzyme. This suggests that calcium binding cannot propagate changes elsewhere unless it does so by through-space (electrostatic) effects.

Table 2.1

Trypsin contacts to PTI:

aa	sas	N	CA	C	O	CB	side	Total
39Y	52	0	1	1	0	4	68	74
40H	17	2	2	6	10	2	5	27
41F	15	5	11	10	13	11	10	60
42C	6	3	3	0	0	2`	6	14
57H	35	0	3	5	5	9	63	85
58C	5	1	2	0	0	0	2	5
60K	36	0	0	0	0	0	11	11
96S	56	0	0	2	8	0	0	10
97N	82	0	5	5	6	1	2	19
98T	49	3	3	2	1	1	1	11
99L	19	1	0	0	0	0	35	36
102D	0	0	0	0	0	0	1	1
151Y	48	0	0	0	0	0	44	44
175Q	48	0	0	0	0	0	5	5
189D	4	0	0	0	1	1	6	8
190S	5	3	3	5	4	3	4	22
191C	8	5	6	8	7	2	0	28
192Q	54	10	16	12	3	15	56	113
193G	20	13	10	7	1	0	0	31
194D	0	8	3	4	0	3	0	18
195S	16	9	8	2	1	9	15	44
213V	4	0	0	1	0	0	5	6
214S	3	2	3	12	13	0	1	31
215W	18	13	15	10	5	13	19	75
216G	31	10	7	6	8	0	0	31
219G	59	1	0	2	3	0	0	6
220C	6	0	2	0	0	0	6	8
226G	1	1	2	1	0	0	0	4
227V	0	1	0	0	0	0	0	1
228Y	1	0	0	0	0	0	1	1

Table 2.1 (cont'd)

PTI contacts to trypsin:

aa	sas	N	CA	C	O	CB	side	Total
11T	19	0	0	0	0	1	5	6
12G	0	0	0	1	2	0	0	3
13P	28	2	10	13	14	12	4	55
14C	38	11	15	12	5	13	19	75
15K	63	18	22	25	24	30	122	241
16A	38	19	21	14	10	19	0	83
17R	48	15	11	12	12	19	82	151
18I	17	7	5	3	0	6	25	46
19I	46	5	4	2	2	6	16	35
34V	16	0	0	0	0	2	7	9
36G	1	1	3	3	5	0	0	12
37G	13	1	7	7	7	0	0	22
38C	15	3	4	0	0	10	9	31
39R	48	1	1	0	0	1	63	66

Table 2.2

Alpha Carbon B Values of the Three Physiological Sites of Trypsin

Structure	Full	Range	Active	Ca++ loop	Autolysis+
1tpo	16.2 ± 4.0 (1.1)	8.9 - 30.7	14.7 ± 2.6	19.3 ± 5.7 (1.3)	20.2 ± 5.5 (1.4) 16.3 ± 4.0 (1.0) 24.2 ± 3.8 (1.6)
3ptn	14.5 ± 6.3 (1.1)	4.6 - 43.4	13.0 ± 4.4	21.6 ± 11.8 (1.7)	22.8 ± 8.6 (1.7) 15.6 ± 3.1 (1.2) 30.0 ± 5.3 (2.3)
3ptb	13.0 ± 4.6 (1.2)	4.1 - 31.1	11.1 ± 2.9	12.9 ± 4.1 (1.2)	18.4 ± 6.7 (1.7) 13.5 ± 3.6 (1.2) 23.4 ± 5.0 (2.3)
1tpp*	9.3 ± 6.6 (1.6)	0.3 - 42.0	6.0 ± 3.3	8.6 ± 5.7 (1.5)	15.0 ± 10.5 (2.5) 7.7 ± 4.6 (1.3) 22.3 ± 9.7 (3.7)
3ptp	9.9 ± 5.1 (1.3)	4.0 - 40.0	7.8 ± 3.6	8.3 ± 3.6 (1.1)	20.1 ± 12.8 (2.6) 10.2 ± 5.6 (1.3) 30.0 ± 9.6 (3.8)
2ptc	24.7 ± 6.1 (1.2)	10.2 - 43.7	20.9 ± 5.2	30.0 ± 5.6 (1.4)	29.5 ± 9.4 (1.4) 21.8 ± 3.6 (1.0) 37.3 ± 6.0 (1.8)
1tpa	19.3 ±5.5 (1.2)	8.1 - 38.0	16.4 ±5.5	23.3 ± 4.3 (1.4)	24.4 ± 10.0 (1.5) 15.7 ± 3.0 (1.0) 33.0 ± 5.2 (2.0)

\+ the first value is the average and standard deviation of P5-P5'
the second value is of P5-P1
the third value is of P1'-P5'

(xx) normalized against active site average (site/active)

* two alpha carbons of 1tpp have no B values reported

Tables 2.3.1 - 2.3.5

Hydrogen Bonding Patterns of the Calcium and Autolysis Loops of Trypsin Crystal Structures

Table 2.3.1

Intra-Calcium Loop

Donor	Acceptor	1tpo	3ptp	1tpp	3ptb	3ptn	2ptc	1tpa
71 N	70 OE					x		
71 N	77 OE		•			•		
72 N	77 OE	x	x	x		x	x	•
72 N	72 OD1			x				
74 N	72 OD1	x			x	x	x	x
75 N	72 O	x	•	•	•	•	•	•
75 N	72 OD1	x		•	•	•	•	
77 N	80 OE	x	x	x	x	x	x	x
78 N	80 OE	•	x	x	x	x	x	x
79 N	80 O						•	
79 N	80 OE	x	•	•			x	x
80 N	80 OE	x	x		x	x	x	x

Table 2.3.2

Calcium Loop - Other Residues

Donor	Acceptor	1tpo	3ptp	1tpp	3ptb	3ptn	2ptc	1tpa
24 N	71 OD			•		•		•
71 N	53 O		x					
72 N	53 O		x					
73 N	153 O	x	x					
74 ND	153 OD	x	•					
117 NH	78 O				•			
117 NH	79 OD	•		•	x			
155 N	71 O			•	•	•	•	•
155 N	71 OD			•	•	•	•	•

Table 2.3.3

Intra-Autolysis Loop

Donor	Acceptor	1tpo	3ptp	1tpp	3ptb	3ptn	2ptc	1tpa
143 ND2	145 O		•	•	•	x		•
143 ND2	148 O	x	x	x	x	x	x	x
144 N	150 O	x	x		x	x	x	x
144 OG1	142 O		•					
144 OG1	150 O	x		x	x	x	x	x
145 N	143 OD	x	x	x	x	x	x	x
145 NZ	144 O		x					
146 N	147 O							x
146 OG	144 O		•					
146 OG	147 OG	•		•	•	•		
147 OG	146 O							x
150 N	143 OD	x	x	x	x	x	x	x

Table 2.3.4

Autolysis Loop - Other Residues

Donor	Acceptor	1tpo	3ptp	1tpp	3ptb	3ptn	2ptc	1tpa
16 N	142 O			•		•		
16 N	143 O	x	x	x	x	x	x	x
16 N	194 OD				x			
142 N	193 OD			•				
142 N	194 OD	x	x	x	x	x	x	x
143 N	192 O	x	x	x	x	x	x	x

Table 2.3.5

Autolysis Loop - Calcium Loop

Donor	Acceptor	1tpo	3ptp	1tpp	3ptb	3ptn	2ptc	1tpa
141 NE1	71 O	x	x	x	x	x	x	x

Figure 2.1
1tpo Active Site

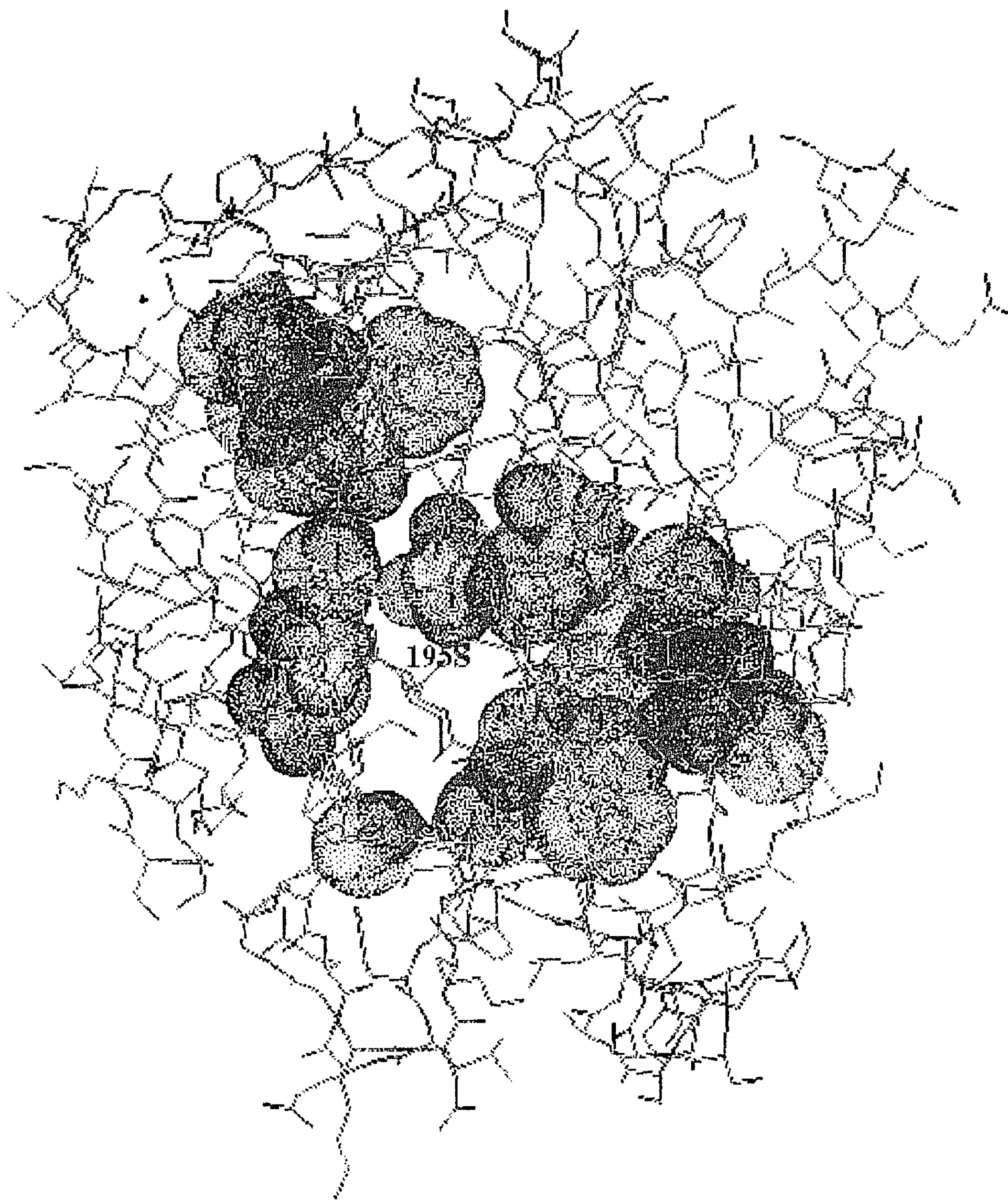


Figure 2.2
Calcium & Autolysis Loops

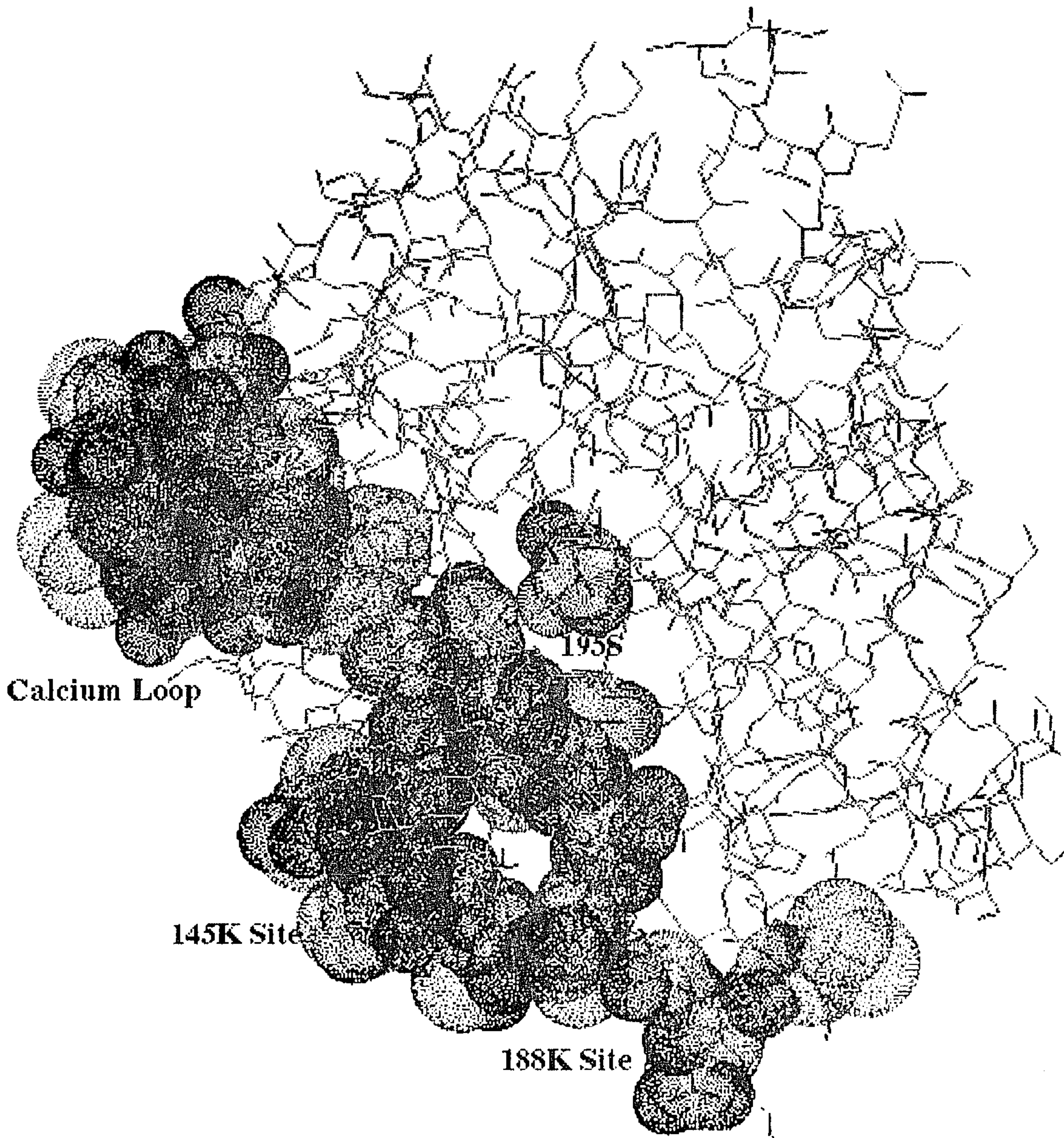
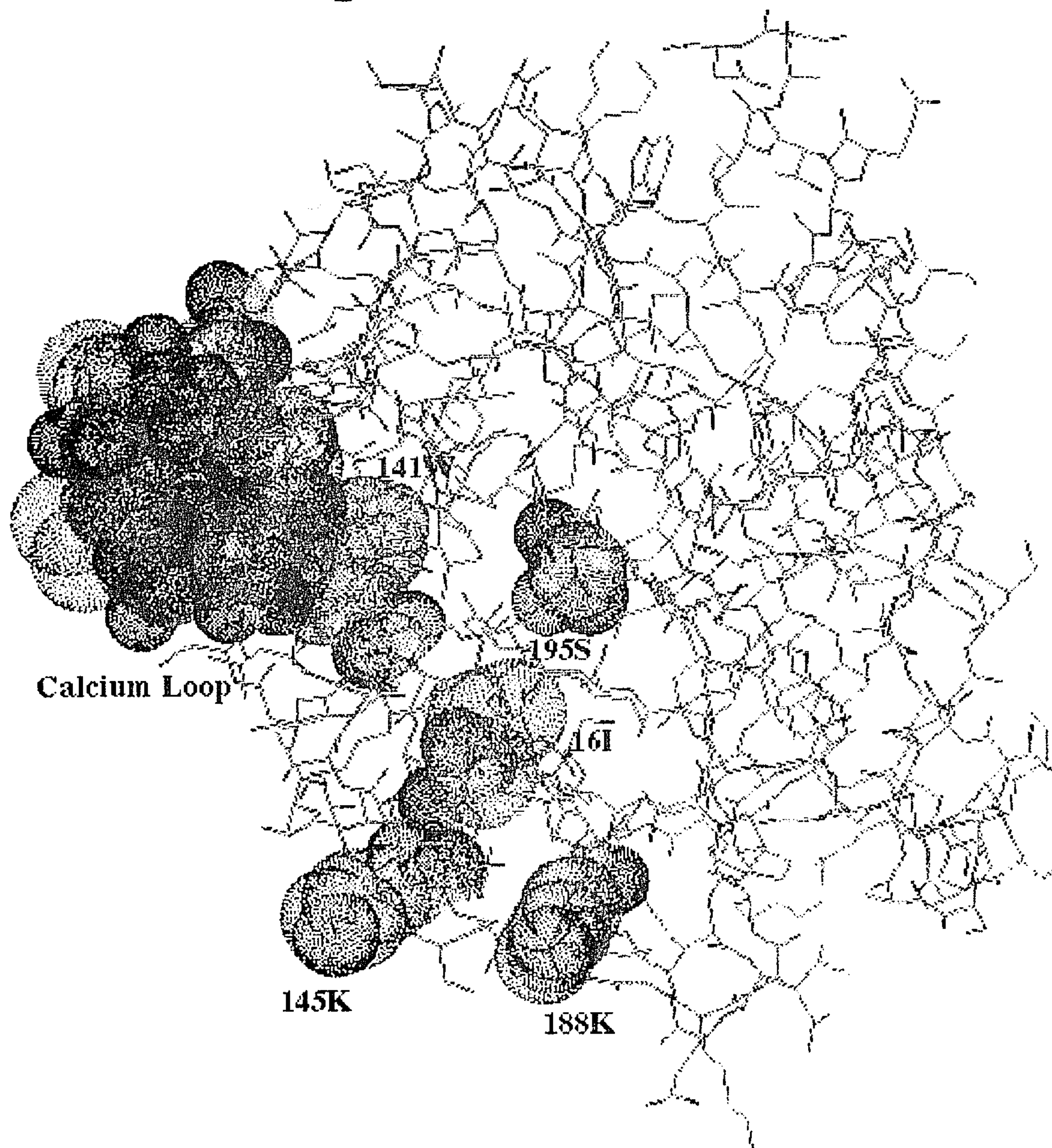


Figure 2.3

Calcium Loop Relationship to Significant Residues



Chapter 3

Modeling of the Michaelis Complex of β-Trypsin Autolysis and Evaluation of the Resulting Backbone Changes

Goals

To model the β-trypsin autolysis dimer by a combination of a rotational screening procedure and CHARMM energy minimizations, and to analyze the 'substrate' autolysis site alpha carbon changes required to form the Michaelis complex.

3.1 Modeling of the Michaelis Complex of β-Trypsin Autolysis

Three analogies were investigated as starting points in the modeling of the Michaelis complex of β-trypsin autolysis. The first required study of the 5cha chymotrypsin crystal structure, found by Blevins & Tulinsky (1985) to crystallize with two molecules comprising the asymmetric unit and with the active sites of each involved in the interaction. The second analyzed the two different unit cell interactions of the bovine β–trypsins included in the library of Chapter 1. Six of the structures crystallize to the same space group - P 21 21 21. Only 3ptn crystallizes to a different space grouping - P 31 2 1. These two groups were analyzed in Chapter 2 for intermolecular interactions, with emphasis put on active and autolysis site interactions. The third explored PTI binding to β–trypsin in the 2ptc structure by utilizing the P5-P5' residues of PTI as templates for the binding of the 'substrate' to the 'enzyme' active site.

3.1.1 Chymotrypsin Crystal Structure Analogy

The residues of the two molecules of the asymmetric unit of the 5cha crystal which are in contact are listed below. The number of vdW contacts (see Methods) is listed in parentheses after the residue identification:

5cha1 residues in contact with 5cha2:

35D (20)	37T (17)	39F (16)	41F (30)	57H (69)	58C (24)
59G (35)	60V (9)	61T (8)	64D (10)	73Q (12)	74G (5)
94Y (1)	96S (13)	97L (1)	99I (18)	145R (7)	146Y (115)
149A (78)	150N (41)	151T (3)	153D (9)	172W (4)	175K (1)
192M (14)	195S (4)	214S (9)	215W (42)	216G (16)	217S (5)
218S (54)	219T (25)				

5cha2 residues in contact with 5cha1:

35D (8)	37T (28)	39F (30)	41F (23)	57H (75)	58C (25)
59G (40)	60V (9)	61T (8)	64D (10)	73Q (6)	74G (1)
94Y (2)	97L (2)	99I (22)	102D (1)	145R (15)	146Y (115)
149A (81)	150N (40)	151T (15)	153D (6)	172W (5)	192M (14)
195S (3)	214S (10)	215W (42)	216G (17)	217S (6)	218S (54)
219T (23)					

The identity of the residues indicates that the molecules are 'face to face', with an almost perfectly symmetric relationship between the two. Furthermore, the active and autolysis sites are involved in the contact with the distances between the 195S OG and 145K C being 9.6 and 9.8 Å. When the 1tpo trypsin structure was superimposed onto both molecules in the 5cha dimer, these distances increased to 10.8 and 10.6 Å.

These large distances, which would require a translation (and possibly rotation) of one of the molecules to form the Michaelis complex, coupled with the fact that in chymotrypsin the 147 and 148 residues are excised, thereby creating a second amino and carboxy terminal, led to the conclusion that the 5cha dimer is an unsatisfactory starting point for modeling trypsin autolysis. No evidence from previously described crystal structure comparisons and B factor analyses, nor molecular dynamics simulations (Brunger, 1987), exists which suggests that individual residues or atoms of trypsin can move on this scale.

3.1.2 Trypsin Crystal Space Analogy

Appendix A lists the crystal contacts of the bovine β-trypsin structures included in library of Chapter 1. 195S and 145K were shown not to form any contacts in either the 1tpo or 3ptn crystals. Below are listed the distances between the active site 195S OG and the autolysis site 145K C of the other molecules which comprise the unit cells of the 1tpo and 3ptn crystals:

Enzyme	Substrate	d(Å)	Enzyme	Substrate	d(Å)
1tpo1	1tpo2	50	3ptn1	3ptn2	30
	1tpo3	33		3ptn3	60
	1tpo4	32		3ptn4	33
	1tpo5	32		3ptn5	57
				3ptn6	38
				3ptn7	52
				3ptn8	50

This analysis shows that the distances between the 195S OG of the 'enzymes' and the C145K of the 'substrates' are too great to be considered as starting points in the modeling of the β-trypsin autolysis dimer. To achieve the Michaelis complex it would be necessary to extensively translate (and possibly rotate) one of the two molecules.

3.1.3 Trypsin/PTI Analogy

This final analogy is based on a simple premise - that the C=O atoms of the peptide bond that is cleaved in conversion of β to α trypsin must be in approximately the same orientation relative to the 195S OG as the 15K residue of PTI in the trypsin/PTI complex. This is an incontrovertible fact because 145K-146S cleavage occurs in β-trypsin (Smith & Shaw, 1969).

Inspection of the autolysis and reactive site primary sequences of trypsin and PTI reveals some interesting differences as well as similarities.

	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'
trypsin (1tpo)	W	G	N	T	K	S	S	G	I	S
PTI (4pti)	T	G	P	C	K	A	R	I	I	R

a. Both have Lys at the P1 position, Gly at P4, and Ile at P4'.

b. An Asn is replaced by a Pro at P3 - this could have stereochemical consequences.

c. A Trp is replaced by a Thr at P5 - Trp is much bulkier.

d. A Ser is replaced by an Arg at P2' - Arg is much larger and charged.

e. A Ser is again replaced by an Arg at P5' - but P5' of PTI does not make any vdW contacts in the complexed structures.

Below are listed distances from the N terminus of the P5 residue to the carbonyl carbon of the five P1'-P5' residues of the seven trypsin and four PTI structures included in the library of Chapter 1:

Trypsins:

	1tpo	3ptb	3ptn	3ptp	1tpp	2ptc	1tpa
P5-P1	11.9	11.9	12.1	11.7	12.0	12.0	11.9
-P1'	14.5	14.6	14.4	14.5	14.5	14.2	14.2
-P2'	16.5	16.6	16.5	16.6	16.4	16.4	16.4
-P3'	15.5	15.5	15.4	15.8	15.6	15.3	15.1
-P4'	12.3	12.4	12.5	12.4	12.4	12.3	12.2
-P5'	10.2	10.2	10.2	10.2	10.2	10.1	10.0

Inhibitors:

	4pti	5pti	2ptc	1tpa
P5-P1	8.7	8.9	8.8	8.9
-P1'	9.7	9.4	9.4	9.5
-P2'	9.6	9.0	9.2	9.1
-P3'	9.5	9.2	9.2	9.2
-P4'	10.1	9.9	9.9	9.9
-P5'	9.7	9.6	9.7	9.6

These results show that the conformation of the trypsin autolysis site and PTI reactive sites are consistent throughout their representative structures. However, the backbone of the autolysis site of β-trypsin is much more 'extended' than the reactive site of the inhibitors as indicated by the P5-P1', P5-P2', and P5-P3' distances. This is, in large part, due to the presence of a proline residue at the P3 subsite in the inhibitors. Nevertheless, the interaction between PTI and trypsin in the 2ptc structure was chosen as the basis for the modeling of the autolysis dimer of β-trypsin autolysis. All subsequent superpositioning equivalence schemes were based on the assumption that some, or all, of the P5-P1 residues align themselves in the active site. This is consistent with trypsin being

an amino peptidase which allows bulk water to enter from the C terminus side in the deacylation step.

3.2 Results

3.2.1 Rotational Screening Results

Four combinations of two 'enzymes' and two 'substrates' were analyzed with the screening procedure outlined in the Methods section. The P2-P1+CD equivalence was chosen for the SP method to generate the initial configuration for the screening analyses. This equivalence was chosen because it generated the least initial steric overlap of the numerous schemes tested and because the CD atom of the lysine sidechain is the furthest atom from the alpha carbon in common with arginine. This also facilitated the basic site analyses of Chapter 5. The screening was done in 2° increments from -30° to 30° around all three axes, except in the 2ptc-3ptp screening, which was extended to 40° around the x axis. Additional screening, in 10° increments, to 90° around all three axes [not shown] was performed to check for minima far from the starting configurations. All screening was done with 'cropped' structures (see Methods), based on the premise that sidechain atoms beyond CB are able to adapt without changing backbone conformations.

The first autolysis dimer modeling utilized 2ptc as the 'enzyme' and 1tpo as the 'substrate'. In the 2ptc crystal structure, the distance between the C195S of trypsin and C15K of PTI is 3.22Å. In the 2ptc-1tpo autolysis dimer modeling, the distance between the C195K of the 'enzyme' and C145K of the 'substrate' is 3.27Å. The initial SP equivalence led to 40 atoms of the 'enzyme' and 34 atoms of the 'substrate' as overlapped. The minimum overlap achieved by the rotational screening method reduced these numbers to 24 in the 'enzyme' and 25 in the 'substrate'. The 'enzyme' and 'substrate' atoms identified as overlapping in those 20 rotations, of the 29,971 sampled, which reduced the overlap of the 'enzyme' to 24 or 25 and the 'substrate' to 25 - 28 are listed in Table 3.1.

The second modeling combination used 3ptp as the 'substrate'. 3ptp was chosen because this crystal structure exhibited the greatest autolysis site conformational variability from the 1tpo structure (see Appendix B). Figure 3.1 shows N, CA, and C backbone atoms of the 3ptp autolysis site superimposed on the 1tpo autolysis site. 3ptp was superimposed onto the 1tpo structure by equivalencing all 223 alpha carbon of the two structures, as was done in Chapter 1. The initial equivalence led to 59 atoms of the 'enzyme' and 58 atoms of the 'substrate' overlapped. The screening range was extended 10° around the x axis because of the finding of minima at x = 30 °. None of the minimum overlapped structures resulted from this extended screening. Seven of the 31,176 rotations sampled reduced the 'enzyme' overlap to 29 atoms. In Table 3.2 are listed those rotations and the 'enzyme' and 'substrate' atoms identified as overlapping from them.

The final two screening analyses were done with 1tpo as the 'enzyme' and the same two substrates. 1tpo was superimposed onto 2ptc by equivalencing all 223 alpha carbon atoms as in Chapter 1. This led to a 195S CB - 145K C distance of 3.28Å in the 1tpo-1tpo dimer.

In the 1tpo-1tpo dimer, the initial configuration had 38 atoms of the 'enzyme' and 31 atoms of the 'substrate' overlapped. This was reduced to 25 or 26 and 25-29, respectively, in 10 rotations. In Table 3.3 are listed those rotations and the 'enzyme' and 'substrate' atoms identified as overlapping.

In the 1tpo-3ptp dimer, the initial configuration had 65 atoms of the 'enzyme' and 58 atoms of the 'substrate' overlapped. This was reduced to 31 and 26-28, respectively, in 5 rotations. In Table 3.4 are listed those rotations and the 'enzyme' and 'substrate' atoms identified as overlapping.

3.2.2 Molecular Mechanics Results

The configurations generated by the rotational screening method of the four combinations of 'enzyme' and 'substrate' were subjected to CHARMM21 minimizations (see Methods), with and without calcium ions on both the 'enzyme' and 'substrate'. These were done in order to analyze the 'substrate' alpha carbon adaptations and 'enzyme' alpha carbon accommodations needed to remove all vdW overlap between the two molecules.

For each of the four combinations, four different constraints were employed, with and without calcium ions present - generating a total of 328 individual minimizations in eight minimization classes. In the first two, no constraints were placed on either the 'enzyme' or 'substrate'. In the second two, the N of the 'enzyme' backbone was constrained. In the third two, the N and C of the 'enzyme' backbone were constrained, while in the fourth all four atoms of the 'enzyme' backbone were constrained. When these minimizations were performed with calciums, the 'enzyme' calcium was also constrained in the three cases where a constraint was employed.

3.2.3 CHARMM Minimization Energies

Table 3.5 lists the average final energies and standard deviations for each of the eight minimization classes of the 2ptc-1tpo dimer. A total of 160 minimizations were performed on the 20 configurations of this 'enzyme' and 'substrate'.

Table 3.6 lists the average final energies and standard deviations for each of the eight minimization classes of the 2ptc-3ptp dimer. A total of 56 minimizations were performed on the 7 configurations of this 'enzyme' and 'substrate'.

Table 3.7 lists the average final energies and standard deviations for each of the eight minimization classes of the 1tpo-1tpo dimer. A total of 80 minimizations were performed on the 10 configurations of this 'enzyme' and 'substrate'.

Table 3.8 lists the average final energies and standard deviations for each of the eight minimization classes of the 1tpo-3ptp dimer. A total of 40 minimizations were performed on the 5 configurations of this 'enzyme' and 'substrate'.

3.2.4 Alpha Carbon Changes Resulting from CHARMM Minimizations

The alpha carbon conformations of the final minimized structures of the 328 dimers were compared to the native crystal structures utilized. Below is summarized the average and standard deviations of the data in Appendices E, F, G, and H. It is of the 13 residues of the 'enzyme' and 15 residues of the 'substrate' predominantly overlapped in the initial autolysis dimers as determined from inspection of Tables 3.1 - 3.4.

The 'enzyme' residues found to predominantly overlap are:

39Y	40H	41F	42C	43G	55A	143N
151Y	192Q	195S	196G	214S	215W	

The 'substrate' residues found to predominantly overlap are:

17V	19G	40H	74N	146S	147S	148G	149T
150S	151Y	152P	186E	188G	221Q	222K	

Above each residue in Appendices E - H are listed the deviation of that residue's alpha carbon based on its B Value and on its SP comparisons with 3ptp (in the 'substrate' changes) and 2ptc (in the 'enzyme' changes). For each comparison are listed the SP results for the full 223 atom comparisons between the minimized 'substrate' and 'enzyme' and the corresponding starting structures.

3.2.4.1 2ptc - 1tpo

Appendix E lists the alpha carbon changes for the 160 minimizations performed on the 2ptc-1tpo autolysis dimer. Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 40 minimizations with no constraints, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.0 ± 0.6	0.9 ± 0.5	1.2 ± 0.9	1.1 ± 0.7
sub02	1.4 ± 1.1	1.4 ± 1.1	1.8 ± 0.9	1.8 ± 0.9
sub03	1.0 ± 0.5	1.1 ± 0.5	1.3 ± 0.6	1.3 ± 0.5
sub04	1.0 ± 0.7	1.0 ± 0.5	1.3 ± 0.7	1.3 ± 0.6
sub05	1.3 ± 0.7	1.4 ± 0.7	2.0 ± 0.8	2.1 ± 0.4
sub06	1.2 ± 0.6	0.9 ± 0.5	1.7 ± 0.7	1.4 ± 0.5
sub07	1.1 ± 0.7	1.1 ± 0.7	1.7 ± 0.7	1.7 ± 0.7
sub08	1.3 ± 0.7	1.2 ± 0.7	1.9 ± 0.7	1.7 ± 0.7
sub09	1.2 ± 0.6	1.2 ± 0.7	1.7 ± 0.6	1.7 ± 0.7
sub10	1.0 ± 0.6	1.1 ± 0.5	1.3 ± 0.8	1.3 ± 0.8
sub11	0.9 ± 0.4	0.9 ± 0.5	1.2 ± 0.6	1.3 ± 0.8
sub12	1.0 ± 0.6	1.1 ± 0.6	1.4 ± 0.7	1.6 ± 0.6
sub13	1.1 ± 0.6	1.0 ± 0.4	1.5 ± 0.7	1.2 ± 0.6
sub14	1.0 ± 0.4	1.0 ± 0.4	1.2 ± 0.5	1.2 ± 0.5
sub15	0.9 ± 0.4	1.5 ± 1.2	1.0 ± 0.5	1.8 ± 0.9
sub16	1.0 ± 0.5	1.0 ± 0.4	1.3 ± 0.6	1.2 ± 0.6
sub17	1.0 ± 0.5	1.0 ± 0.4	1.3 ± 0.3	1.2 ± 0.3
sub18	1.0 ± 0.5	1.0 ± 0.6	1.2 ± 0.6	1.3 ± 0.7
sub19	0.9 ± 0.5	1.1 ± 0.5	1.2 ± 0.6	1.4 ± 0.7
sub20	1.0 ± 0.5	1.0 ± 0.5	1.2 ± 0.7	1.2 ± 0.8

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 40 minimizations with no constraints, with and without calcium:

	13 residues	w/ Calciums
sub01	1.0 ± 0.3	1.0 ± 0.4
sub02	0.9 ± 0.3	0.9 ± 0.3
sub03	1.1 ± 0.4	1.1 ± 0.4
sub04	0.8 ± 0.4	0.9 ± 0.5
sub05	0.9 ± 0.3	0.9 ± 0.3
sub06	0.8 ± 0.3	0.9 ± 0.4
sub07	0.9 ± 0.4	0.7 ± 0.4
sub08	1.0 ± 0.4	0.9 ± 0.4
sub09	0.9 ± 0.4	0.9 ± 0.3
sub10	1.0 ± 0.5	0.9 ± 0.3
sub11	0.9 ± 0.5	0.9 ± 0.3
sub12	0.8 ± 0.3	0.9 ± 0.4
sub13	0.9 ± 0.3	1.0 ± 0.4
sub14	1.0 ± 0.4	1.0 ± 0.4
sub15	1.1 ± 0.2	1.1 ± 0.3
sub16	1.0 ± 0.2	1.0 ± 0.4
sub17	1.0 ± 0.3	1.0 ± 0.4
sub18	0.9 ± 0.3	1.0 ± 0.3
sub19	0.9 ± 0.3	1.0 ± 0.3
sub20	1.0 ± 0.3	1.0 ± 0.3

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 40 minimizations with the backbone N constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.5 ± 1.0	1.4 ± 0.9	2.3 ± 1.2	2.1 ± 1.1
sub02	1.5 ± 0.9	1.6 ± 0.9	2.0 ± 1.2	2.1 ± 1.2
sub03	1.5 ± 0.8	1.7 ± 0.9	2.0 ± 0.7	2.4 ± 0.9
sub04	1.4 ± 0.9	2.0 ± 1.2	2.1 ± 0.9	2.9 ± 1.4
sub05	1.6 ± 0.9	1.9 ± 1.1	2.4 ± 0.8	3.1 ± 1.8
sub06	1.7 ± 1.3	1.7 ± 1.2	2.8 ± 1.4	2.6 ± 1.4
sub07	1.3 ± 0.8	1.5 ± 0.9	1.9 ± 1.1	2.4 ± 0.8
sub08	2.1 ± 1.4	1.9 ± 1.2	3.3 ± 1.5	2.9 ± 1.3
sub09	1.7 ± 1.2	1.9 ± 1.3	2.8 ± 1.4	3.1 ± 1.5
sub10	1.6 ± 0.9	1.1 ± 0.6	2.4 ± 1.0	1.6 ± 0.6
sub11	1.5 ± 0.9	1.6 ± 1.0	2.2 ± 1.1	2.5 ± 1.0
sub12	1.6 ± 1.1	1.8 ± 1.1	2.4 ± 1.2	2.7 ± 1.1
sub13	1.7 ± 1.0	1.4 ± 0.8	2.5 ± 0.9	1.8 ± 1.2
sub14	1.7 ± 1.0	1.7 ± 0.9	2.6 ± 0.8	2.6 ± 0.8
sub15	1.6 ± 0.9	1.3 ± 0.9	2.0 ± 1.3	1.6 ± 1.5
sub16	1.6 ± 0.9	1.6 ± 0.8	2.1 ± 1.1	2.1 ± 1.0
sub17	1.7 ± 0.9	1.6 ± 0.8	2.4 ± 0.9	2.2 ± 0.9
sub18	2.0 ± 1.1	1.8 ± 1.1	3.0 ± 0.7	2.7 ± 1.0
sub19	1.0 ± 0.7	1.4 ± 0.8	1.7 ± 0.7	2.2 ± 0.8
sub20	1.5 ± 0.9	1.7 ± 1.0	2.1 ± 1.2	2.7 ± 1.0

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 40 minimizations with the backbone N constrained, with and without calcium:

	13 residues	w/ Calciums
sub01	0.2 ± 0.1	0.2 ± 0.2
sub02	0.2 ± 0.1	0.2 ± 0.1
sub03	0.2 ± 0.1	0.2 ± 0.1
sub04	0.3 ± 0.2	0.1 ± 0.1
sub05	0.3 ± 0.3	0.1 ± 0.1
sub06	0.2 ± 0.1	0.2 ± 0.1
sub07	0.2 ± 0.2	0.2 ± 0.1
sub08	0.1 ± 0.1	0.1 ± 0.1
sub09	0.1 ± 0.1	0.1 ± 0.1
sub10	0.2 ± 0.1	0.3 ± 0.2
sub11	0.2 ± 0.1	0.2 ± 0.1
sub12	0.2 ± 0.1	0.2 ± 0.1
sub13	0.2 ± 0.1	0.2 ± 0.1
sub14	0.2 ± 0.1	0.2 ± 0.1
sub15	0.2 ± 0.1	0.2 ± 0.1
sub16	0.2 ± 0.1	0.2 ± 0.1
sub17	0.2 ± 0.1	0.2 ± 0.1
sub18	0.2 ± 0.1	0.2 ± 0.1

sub19	0.2 ± 0.2	0.2 ± 0.1
sub20	0.2 ± 0.1	0.1 ± 0.1

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 40 minimizations with the backbone N and C constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.6 ± 1.0	1.6 ± 1.0	2.5 ± 1.1	2.6 ± 1.0
sub02	1.8 ± 1.0	1.6 ± 0.8	2.6 ± 1.0	2.2 ± 0.6
sub03	1.4 ± 0.9	1.8 ± 0.9	2.1 ± 1.2	2.7 ± 0.7
sub04	1.9 ± 1.3	2.0 ± 1.2	3.2 ± 0.9	3.0 ± 1.1
sub05	1.5 ± 1.2	1.8 ± 1.1	2.6 ± 1.0	2.8 ± 0.9
sub06	1.7 ± 1.1	1.5 ± 1.0	2.5 ± 1.2	2.3 ± 1.1
sub07	1.6 ± 0.9	1.7 ± 1.0	2.2 ± 1.0	2.7 ± 0.8
sub08	2.0 ± 1.1	2.0 ± 1.1	3.0 ± 1.1	3.0 ± 1.0
sub09	1.6 ± 1.1	1.7 ± 1.1	2.5 ± 1.2	2.8 ± 1.1
sub10	1.7 ± 1.0	1.6 ± 1.0	2.5 ± 1.2	2.4 ± 1.3
sub11	1.3 ± 0.8	1.5 ± 0.9	2.0 ± 0.9	2.3 ± 1.1
sub12	1.4 ± 1.1	1.6 ± 1.0	2.4 ± 1.2	2.4 ± 1.1
sub13	1.6 ± 1.0	1.6 ± 0.9	2.3 ± 1.2	2.2 ± 1.0
sub14	1.2 ± 0.7	1.0 ± 0.4	1.8 ± 0.7	1.2 ± 0.5
sub15	1.6 ± 0.9	1.8 ± 1.1	2.3 ± 0.9	2.7 ± 0.8
sub16	1.6 ± 0.9	1.4 ± 0.8	2.2 ± 1.1	2.0 ± 1.0
sub17	1.8 ± 1.0	1.9 ± 0.9	2.7 ± 0.7	2.9 ± 0.6
sub18	1.6 ± 0.8	1.7 ± 0.9	2.2 ± 0.9	2.4 ± 0.8
sub19	1.7 ± 0.9	1.2 ± 0.7	2.6 ± 0.6	1.9 ± 0.6
sub20	1.6 ± 1.0	1.5 ± 1.0	2.3 ± 1.2	2.2 ± 1.2

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 40 minimizations with the backbone N and C constrained, with and without calcium:

	13 residues	w/ Calciums
sub01	0.1 ± 0.1	0.1 ± 0.1
sub02	0.1 ± 0.1	0.1 ± 0.1
sub03	0.2 ± 0.1	0.1 ± 0.1
sub04	0.4 ± 0.2	0.1 ± 0.1
sub05	0.4 ± 0.2	0.1 ± 0.1
sub06	0.4 ± 0.2	0.1 ± 0.1
sub07	0.4 ± 0.2	0.1 ± 0.1
sub08	0.1 ± 0.1	0.1 ± 0.1
sub09	0.1 ± 0.1	0.1 ± 0.1
sub10	0.1 ± 0.1	0.1 ± 0.1
sub11	0.2 ± 0.2	0.1 ± 0.1
sub12	0.1 ± 0.1	0.1 ± 0.1
sub13	0.1 ± 0.1	0.1 ± 0.1
sub14	0.2 ± 0.2	1.1 ± 0.4
sub15	0.1 ± 0.1	0.1 ± 0.1
sub16	0.1 ± 0.1	0.2 ± 0.2

sub17	0.1 ± 0.1	0.1 ± 0.1
sub18	0.1 ± 0.1	0.1 ± 0.1
sub19	0.1 ± 0.1	0.2 ± 0.2
sub20	0.1 ± 0.1	0.1 ± 0.1

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 40 minimizations with the backbone constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.4 ± 0.9	1.6 ± 0.9	2.2 ± 0.9	2.3 ± 1.0
sub02	0.9 ± 0.6	2.0 ± 1.0	1.6 ± 0.3	2.4 ± 0.8
sub03	1.7 ± 0.9	1.8 ± 1.0	2.5 ± 0.8	2.8 ± 0.9
sub04	1.8 ± 1.1	2.0 ± 1.5	2.7 ± 1.0	3.3 ± 1.6
sub05	2.1 ± 1.2	1.8 ± 1.2	3.4 ± 0.9	3.0 ± 1.2
sub06	1.5 ± 1.0	1.7 ± 1.1	2.4 ± 1.1	2.8 ± 1.0
sub07	1.6 ± 1.1	1.6 ± 1.0	2.7 ± 1.0	2.7 ± 1.0
sub08	2.2 ± 1.4	1.9 ± 1.2	3.5 ± 1.1	2.9 ± 1.1
sub09	2.2 ± 1.4	1.8 ± 1.2	3.7 ± 1.1	2.8 ± 1.2
sub10	1.9 ± 1.2	1.5 ± 0.9	2.9 ± 1.2	2.3 ± 0.9
sub11	1.8 ± 1.1	1.0 ± 0.8	2.7 ± 1.2	1.9 ± 0.5
sub12	1.7 ± 1.2	1.8 ± 1.2	2.6 ± 1.3	2.7 ± 1.2
sub13	1.5 ± 0.9	1.6 ± 0.9	2.2 ± 1.0	2.2 ± 1.0
sub14	1.7 ± 1.0	1.5 ± 0.9	2.6 ± 1.1	2.1 ± 0.9
sub15	1.7 ± 0.9	1.5 ± 0.9	2.4 ± 0.8	2.2 ± 0.9
sub16	1.8 ± 1.1	1.9 ± 1.1	2.7 ± 0.5	2.9 ± 1.0
sub17	1.9 ± 1.2	1.9 ± 1.2	3.1 ± 1.0	3.1 ± 1.2
sub18	1.6 ± 0.9	1.7 ± 1.0	2.5 ± 0.7	2.6 ± 0.7
sub19	1.5 ± 0.9	1.4 ± 0.9	2.3 ± 0.6	2.4 ± 0.6
sub20	1.7 ± 1.0	1.9 ± 1.1	2.5 ± 0.8	2.9 ± 1.0

Figure 3.2 shows the N, CA, and C backbone atoms of the final minimized structure of subrot17 (rotation = 12, 0, 22), without calciums and with the backbone of the 'enzyme' constrained during the minimization, superimposed on the starting structure. This figure highlights the changes to the P1'-P5' residues (SP rms deviation = 1.9 ± 1.2).

3.2.4.2 2ptc - 3ptp

Appendix F lists the alpha carbon changes for the 56 minimizations performed on the 2ptc-3ptp autolysis dimer. Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 14 minimizations with no constraints, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.2 ± 0.6	1.2 ± 0.5	1.8 ± 0.6	1.7 ± 0.6
sub02	0.9 ± 0.6	1.2 ± 0.5	1.4 ± 0.4	1.6 ± 0.4
sub03	1.1 ± 0.5	1.2 ± 0.5	1.6 ± 0.5	1.6 ± 0.4
sub04	1.1 ± 0.6	1.2 ± 0.7	1.6 ± 0.8	1.7 ± 0.9
sub05	1.1 ± 0.5	1.1 ± 0.5	1.5 ± 0.5	1.4 ± 0.4
sub06	1.0 ± 0.6	1.0 ± 0.6	1.3 ± 0.6	1.3 ± 0.6
sub07	0.9 ± 0.5	0.9 ± 0.5	1.4 ± 0.6	1.4 ± 0.5

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 14 minimizations with no constraints, with and without calcium:

	13 residues	w/ Calciums
sub01	1.3 ± 0.7	1.3 ± 0.8
sub02	0.9 ± 0.3	1.0 ± 0.4
sub03	1.0 ± 0.4	1.1 ± 0.4
sub04	1.0 ± 0.3	1.1 ± 0.3
sub05	1.0 ± 0.4	1.0 ± 0.4
sub06	0.9 ± 0.3	0.9 ± 0.4
sub07	1.1 ± 0.5	0.9 ± 0.4

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 14 minimizations with the backbone N constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.4 ± 0.6	1.2 ± 0.7	2.1 ± 0.6	1.9 ± 0.5
sub02	1.5 ± 0.9	1.6 ± 1.1	2.4 ± 0.8	2.7 ± 1.1
sub03	1.7 ± 1.0	1.8 ± 1.1	2.6 ± 0.8	2.8 ± 1.0
sub04	1.6 ± 1.0	1.7 ± 1.1	2.7 ± 1.2	2.8 ± 1.3
sub05	1.5 ± 1.0	1.7 ± 0.9	2.6 ± 0.6	2.6 ± 0.6
sub06	1.5 ± 0.9	1.5 ± 0.9	2.4 ± 0.8	2.4 ± 0.9
sub07	1.2 ± 0.7	1.2 ± 0.8	1.9 ± 0.8	2.0 ± 0.8

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 14 minimizations with the backbone N constrained, with and without calcium:

	13 residues	w/ Calciums
sub01	0.2 ± 0.3	0.2 ± 0.2
sub02	0.2 ± 0.1	0.2 ± 0.1
sub03	0.2 ± 0.1	0.2 ± 0.1
sub04	0.2 ± 0.1	0.2 ± 0.1
sub05	0.2 ± 0.1	0.2 ± 0.1
sub06	0.2 ± 0.1	0.2 ± 0.1
sub07	0.3 ± 0.3	0.4 ± 0.3

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 14 minimizations with the backbone N and C constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.8 ± 1.4	1.7 ± 1.4	3.2 ± 1.8	3.1 ± 1.7
sub02	1.5 ± 1.1	1.7 ± 1.0	2.5 ± 1.3	2.6 ± 1.1
sub03	1.9 ± 1.3	2.0 ± 1.3	3.0 ± 1.5	3.1 ± 1.6
sub04	1.9 ± 1.3	1.9 ± 1.4	3.1 ± 1.6	3.2 ± 1.7
sub05	1.7 ± 1.1	1.7 ± 1.0	2.8 ± 0.9	2.8 ± 0.9
sub06	1.8 ± 1.1	1.9 ± 1.2	2.7 ± 1.0	2.9 ± 1.3
sub07	1.6 ± 1.1	1.7 ± 1.0	2.8 ± 0.9	2.8 ± 0.9

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 14 minimizations with the backbone N and C constrained, with and without calcium:

	13 residues	w/ Calciums
sub01	0.1 ± 0.1	0.1 ± 0.1
sub02	0.1 ± 0.1	0.1 ± 0.1
sub03	0.1 ± 0.1	0.1 ± 0.1
sub04	0.1 ± 0.1	0.1 ± 0.1
sub05	0.1 ± 0.1	0.1 ± 0.1
sub06	0.1 ± 0.2	0.1 ± 0.1
sub07	0.2 ± 0.2	0.2 ± 0.2

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 14 minimizations with the backbone constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.5 ± 1.2	1.9 ± 1.4	2.7 ± 1.2	3.2 ± 1.6
sub02	1.9 ± 1.3	1.9 ± 1.2	3.1 ± 1.4	3.0 ± 1.1
sub03	2.0 ± 1.7	2.0 ± 1.3	3.4 ± 1.5	3.1 ± 1.6
sub04	2.3 ± 1.7	1.9 ± 1.4	4.0 ± 1.8	3.2 ± 1.7
sub05	1.7 ± 0.9	1.7 ± 1.0	2.7 ± 0.5	2.8 ± 0.9
sub06	0.7 ± 0.6	1.9 ± 1.2	1.2 ± 0.4	2.9 ± 1.3
sub07	1.3 ± 0.7	1.6 ± 1.1	2.0 ± 0.5	2.8 ± 0.9

3.2.4.3 1tpo - 1tpo

Appendix G lists the alpha carbon changes for the 80 minimizations performed on the 1tpo-1tpo autolysis dimer. Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 20 minimizations with no constraints, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.0 ± 0.5	1.0 ± 0.5	1.5 ± 0.5	1.3 ± 0.7
sub02	1.1 ± 0.7	1.2 ± 0.7	1.6 ± 0.8	1.7 ± 0.8
sub03	1.1 ± 0.6	1.2 ± 0.6	1.7 ± 0.7	1.7 ± 0.7
sub04	1.1 ± 0.7	1.2 ± 0.6	1.3 ± 0.9	1.8 ± 0.3
sub05	1.3 ± 0.8	1.2 ± 0.6	1.9 ± 0.6	1.6 ± 0.6
sub06	1.0 ± 0.5	1.0 ± 0.5	1.2 ± 0.7	1.2 ± 0.6
sub07	1.1 ± 0.5	1.3 ± 0.7	1.5 ± 0.5	1.9 ± 0.8
sub08	1.0 ± 0.6	0.8 ± 0.6	1.4 ± 0.6	1.3 ± 0.6
sub09	1.0 ± 0.5	1.1 ± 0.5	1.2 ± 0.6	1.2 ± 0.6
sub10	1.1 ± 0.6	1.1 ± 0.6	1.5 ± 0.6	1.5 ± 0.6

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 20 minimizations with no constraints, with and without calcium:

	13 residues	w/ Calciums
sub01	0.9 ± 0.4	0.8 ± 0.4
sub02	0.8 ± 0.4	0.7 ± 0.3
sub03	0.9 ± 0.3	0.8 ± 0.4
sub04	0.9 ± 0.4	0.8 ± 0.4
sub05	0.9 ± 0.4	0.8 ± 0.5
sub06	0.8 ± 0.4	0.8 ± 0.4
sub07	0.8 ± 0.4	0.8 ± 0.4
sub08	0.8 ± 0.4	0.8 ± 0.4
sub09	0.9 ± 0.5	0.9 ± 0.5
sub10	0.9 ± 0.5	0.8 ± 0.5

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 20 minimizations with the backbone N constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.8 ± 1.2	1.7 ± 1.0	3.0 ± 0.9	2.7 ± 0.9
sub02	1.7 ± 0.9	1.6 ± 0.9	2.7 ± 0.4	2.6 ± 0.3
sub03	1.5 ± 0.9	1.6 ± 0.9	2.4 ± 0.8	2.4 ± 0.8
sub04	1.8 ± 1.2	1.9 ± 1.3	2.9 ± 1.0	3.1 ± 1.4
sub05	1.7 ± 1.0	1.8 ± 1.1	2.7 ± 0.6	2.9 ± 0.7

sub06	1.8 ± 1.0	1.6 ± 1.0	2.8 ± 0.7	2.4 ± 0.8
sub07	1.2 ± 0.8	1.5 ± 0.8	1.8 ± 0.8	2.4 ± 0.3
sub08	1.7 ± 1.2	1.5 ± 1.1	2.9 ± 1.0	2.4 ± 1.0
sub09	1.6 ± 1.0	1.6 ± 0.9	2.4 ± 1.0	2.4 ± 0.8
sub10	1.9 ± 1.1	1.7 ± 1.0	2.9 ± 0.8	2.7 ± 0.6

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 20 minimizations with the backbone N constrained, with and without calcium:

	13 residues	w/ Calciums
sub01	0.2 ± 0.1	0.2 ± 0.1
sub02	0.3 ± 0.2	0.3 ± 0.2
sub03	0.2 ± 0.1	0.2 ± 0.1
sub04	0.1 ± 0.1	0.2 ± 0.1
sub05	0.1 ± 0.1	0.1 ± 0.1
sub06	0.2 ± 0.1	0.2 ± 0.1
sub07	0.3 ± 0.2	0.3 ± 0.2
sub08	0.2 ± 0.1	0.2 ± 0.1
sub09	0.2 ± 0.2	0.2 ± 0.2
sub10	0.2 ± 0.1	0.2 ± 0.1

Below is summarized the individual 'substrate' alpha carbon deviation average and standard deviations of the 15 overlapping residues and the P1'-P5' for the 20 minimizations with the backbone N and C constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.6 ± 1.3	1.4 ± 1.1	2.8 ± 1.2	2.4 ± 1.2
sub02	1.7 ± 1.1	1.8 ± 1.1	2.9 ± 0.6	2.9 ± 0.7
sub03	2.0 ± 1.4	2.1 ± 1.3	3.3 ± 1.2	3.3 ± 1.2
sub04	1.7 ± 1.2	1.8 ± 1.2	2.8 ± 1.2	3.0 ± 1.2
sub05	1.5 ± 0.9	1.7 ± 1.0	2.4 ± 0.6	2.8 ± 0.3
sub06	1.8 ± 1.1	1.9 ± 1.1	2.7 ± 0.7	2.9 ± 0.7
sub07	1.7 ± 1.1	1.6 ± 1.1	2.8 ± 1.0	2.8 ± 1.0
sub08	1.6 ± 1.2	1.6 ± 1.2	2.7 ± 1.5	2.7 ± 1.5
sub09	1.6 ± 1.1	1.6 ± 1.1	2.5 ± 1.2	2.5 ± 1.2
sub10	1.8 ± 1.0	1.8 ± 1.0	2.6 ± 0.9	2.6 ± 0.9

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 20 minimizations with the backbone N and C constrained, with and without calcium:

	13 residues	w/ Calciums
sub01	0.1 ± 0.1	0.2 ± 0.1
sub02	0.1 ± 0.1	0.1 ± 0.1
sub03	0.1 ± 0.1	0.1 ± 0.1
sub04	0.1 ± 0.1	0.1 ± 0.1
sub05	0.1 ± 0.1	0.1 ± 0.1

sub06	0.2 ± 0.2	0.1 ± 0.1
sub07	0.1 ± 0.1	0.1 ± 0.1
sub08	0.1 ± 0.1	0.1 ± 0.1
sub09	0.2 ± 0.2	0.2 ± 0.2
sub10	0.1 ± 0.1	0.1 ± 0.1

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 20 minimizations with the backbone constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	2.2 ± 1.6	2.1 ± 1.5	3.8 ± 1.4	3.9 ± 1.1
sub02	1.4 ± 1.0	2.0 ± 1.4	2.3 ± 0.9	3.5 ± 0.9
sub03	1.6 ± 1.2	1.5 ± 1.1	2.8 ± 1.4	2.6 ± 1.2
sub04	1.7 ± 1.3	2.2 ± 1.4	2.9 ± 1.2	3.6 ± 1.2
sub05	2.1 ± 1.3	1.6 ± 1.2	3.4 ± 0.9	2.7 ± 1.3
sub06	1.9 ± 1.3	1.9 ± 1.3	3.2 ± 0.9	3.3 ± 0.7
sub07	2.3 ± 1.3	1.9 ± 1.2	3.5 ± 0.8	3.2 ± 0.6
sub08	1.9 ± 1.5	1.8 ± 1.4	3.4 ± 1.5	3.1 ± 1.7
sub09	1.9 ± 1.2	1.9 ± 1.1	2.9 ± 0.8	2.9 ± 0.7
sub10	2.2 ± 1.6	1.8 ± 1.4	3.8 ± 1.4	2.8 ± 1.9

3.2.4.4 1tpo - 3ptp

Appendix H lists the alpha carbon changes for the 40 minimizations performed on the 1tpo-3ptp autolysis dimer. Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 10 minimizations with no constraints, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.1 ± 0.6	1.0 ± 0.5	1.7 ± 0.4	1.5 ± 0.5
sub02	1.0 ± 0.6	1.2 ± 0.6	1.6 ± 0.5	1.8 ± 0.7
sub03	1.3 ± 0.8	1.1 ± 0.7	1.5 ± 0.6	1.4 ± 0.5
sub04	1.6 ± 0.9	1.3 ± 0.9	2.1 ± 0.7	1.8 ± 0.5
sub05	1.2 ± 0.7	1.1 ± 0.6	1.7 ± 0.6	1.5 ± 0.5

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 10 minimizations with no constraints, with and without calcium:

	13 residues	w/ Calciums
sub01	1.1 ± 0.4	1.0 ± 0.4
sub02	0.9 ± 0.4	1.0 ± 0.4
sub03	1.3 ± 0.8	1.1 ± 0.7
sub04	1.0 ± 0.6	0.9 ± 0.6
sub05	0.9 ± 0.5	0.9 ± 0.5

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 10 minimizations with the backbone N constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.5 ± 0.9	1.2 ± 0.9	2.5 ± 0.9	1.9 ± 1.2
sub02	1.7 ± 1.1	1.9 ± 1.0	2.7 ± 1.1	3.0 ± 0.5
sub03	2.1 ± 1.4	1.9 ± 1.3	3.4 ± 1.1	3.2 ± 0.9
sub04	1.8 ± 1.2	2.1 ± 1.4	2.9 ± 0.5	3.5 ± 0.6
sub05	1.7 ± 0.9	1.7 ± 0.9	2.4 ± 0.7	2.4 ± 0.7

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 10 minimizations with the backbone N constrained, with and without calcium:

	13 residues	w/ Calciums
sub01	0.2 ± 0.2	0.2 ± 0.2
sub02	0.3 ± 0.3	0.2 ± 0.1
sub03	0.2 ± 0.2	0.2 ± 0.2
sub04	0.2 ± 0.2	0.2 ± 0.2
sub05	0.3 ± 0.2	0.3 ± 0.2

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 10 minimizations with the backbone N and C constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	2.2 ± 1.6	2.1 ± 1.5	3.8 ± 1.3	3.7 ± 1.2
sub02	2.0 ± 1.2	2.0 ± 1.2	2.9 ± 1.5	3.4 ± 0.6
sub03	1.8 ± 1.2	1.8 ± 1.2	3.0 ± 0.6	3.0 ± 0.6
sub04	1.9 ± 1.4	1.9 ± 1.3	3.3 ± 0.9	3.3 ± 0.8
sub05	2.0 ± 1.4	1.9 ± 1.3	3.5 ± 0.8	3.3 ± 0.9

Below is summarized the individual 'enzyme' alpha carbon deviation averages and standard deviations of the 13 overlapping residues for the 10 minimizations with the backbone N and C constrained, with and without calcium:

	13 residues	w/ Calciums
sub01	0.1 ± 0.1	0.1 ± 0.1
sub02	0.1 ± 0.1	0.1 ± 0.1
sub03	0.2 ± 0.2	0.2 ± 0.2
sub04	0.2 ± 0.2	0.2 ± 0.2
sub05	0.2 ± 0.2	0.2 ± 0.2

Below is summarized the individual 'substrate' alpha carbon deviation averages and standard deviations of the 15 overlapping residues and the P1'-P5' for the 10 minimizations with the backbone constrained, with and without calcium:

	15 residues	w/ Calciums	P1'-P5'	w/ Calciums
sub01	1.4 ± 1.1	1.4 ± 1.1	2.6 ± 0.8	2.5 ± 1.3
sub02	1.2 ± 0.9	1.2 ± 0.9	2.0 ± 1.0	2.0 ± 1.0
sub03	0.9 ± 0.9	0.9 ± 0.8	1.8 ± 0.5	1.7 ± 0.5
sub04	0.9 ± 0.8	1.0 ± 0.9	1.8 ± 0.6	1.9 ± 0.6
sub05	1.2 ± 1.0	1.1 ± 1.0	2.2 ± 0.9	1.8 ± 1.3

3.3 Discussion

Use of the 2ptc crystal position of PTI in the active site of trypsin as the starting point in the modeling of the β-trypsin autolysis dimer was shown to be superior to the two other starting points which were investigated - the chymotrypsin dimer and trypsin crystal space analogies. Even so, this proved to be inadequate to achieve the 'best fit', defined as having the least steric overlap, between 'enzyme' and 'substrate'. This is due to the sensitivity of the SP method to the conformation of the peptide bonds around the P1 sites. Because only seven atoms were used in the equivalence, small variations in backbone dihedral angles can lead to large variations in the initial orientation of the 'substrate'.

The screening procedure essentially reduces the problem to a translation to the desired peptide bond C=O position, followed by a rigid body conformational search about all three cartesian axes. The approach taken here, however, differs from a pure 'shot gun' approach in that the initial orientation is closer to the 'best' configuration than the vast majority of random orientations are. This statement is supported by the screening results which showed that the minima occur near the initial configuration. It also obviates searching 360° axes space and thus considerably reduces the computational time required for the analyses.

The use of a rotational screening method and subsequent molecular mechanics energy minimizations to alleviate the steric overlap is very similar in philosophy to the

method employed by Wodak (1978) in an analysis of the trypsin/PTI interaction. This study also tested all possible rotational modes of interaction but based the definition of 'best fit' on a non-bonded interaction energy. It was also proposed, but not performed, that unacceptable overlap would be removed for evaluation of the best final fit.

The conformational variabilities of the active, calcium, and autolysis sites (see Chapter 2) became the basis for establishing constraints in the molecular mechanics minimizations employed to remove the steric overlap between the 'enzyme' and 'substrate'. The SP results of the trypsin comparisons in Chapter 2 indicate that the active site alpha carbons move on the order of 0.3 Å.

With this in mind, four different constraints were placed on the 'enzyme' backbone during the minimizations. The results show that the N constraint resulted in 'enzyme' alpha carbon movements closest to the changes observed in the PTI-inhibited structures. The individual 'enzyme' alpha carbon deviation averages and standard deviations of the 15 overlapping residues in the 1tpo-3ptp, 1tpo-3ptb, 1tpo-2ptc, and 1tpo-1tpa SP comparisons (see Table 2.3) are 0.11 ± 0.06, 0.11 ± 0.08, 0.27 ± 0.09, and 0.23 ± 0.09, respectively. The N and C constraint was also found to result in similar 'enzyme' changes. However, the other two constraint schemes are shown to be unrealistic. The minimizations performed with no constraints lead to 'enzyme' alpha carbon changes greater than 1 Å. This is larger than any changes found in the trypsin comparisons of Chapter 2. This, coupled with the fact that the active site alpha carbons have the lowest B values of the three physiological sites, suggests that 1 Å is too great an accommodation for the active site residues to undergo. Conversely, constraining all four backbone atoms of the 223 amino acids of the 'enzyme' is unrealistic because it is too stringent and allows no accommodation, whatsoever, with 'substrate' binding. This constraint also lead to significantly greater movement in the 'substrate' and higher (less negative) minimization energies.

The minimization results also lead to several other significant conclusions. First, the introduction of calcium ions on both 'enzyme' and 'substrate' had no effect on the magnitude of the backbone changes in either the 'enzyme' or 'substrate' in all 42 configurations. Assuming that the potentials used are accurate, this is evidence against calcium having an effect on the 'adaptability' of the autolysis site (see Chapter 6).

Second, the use of native, PTI-inhibited, or DIP-inhibited trypsin as the 'enzyme' or 'substrate' had no effect on the magnitude of backbone changes of either molecule. The use of these four combinations of 'enzyme' and 'substrate' was premised on the fact that the 1tpo - 2ptc SP comparison (see Appendix B) showed the largest active site variability, while the 1tpo - 3ptp SP comparison showed the largest autolysis site variability, as previously shown by Liebman (1986). Other combinations [not shown] also resulted in the same magnitude of alpha carbon changes to the 'enzyme' and 'substrate' and indicates that the final modeled Michaelis complexes are [relatively] independent of the crystal structures used. This agrees with the observation of Wright (1977) that there is no required P2-P2' conformation for trypsin substrates.

Third, the 42 starting configurations of the four 'enzyme' and 'substrate' combinations used were all shown to result in the same magnitude of alpha carbon changes. This is important in that it shows that the best configurations arrived at by the rotational screening method are all equivalent based on the observation that they lead to the same magnitude of backbone changes in the dimerization process.

In lieu of energy minimizations, an attempt was made to alleviate the overlap between the 'enzyme' and 'substrate' of the 2ptc-1tpo dimer by changing the position of the autolysis loop [not shown]. This was accomplished by rigid body rotations of the P5-P5' residues by changing the torsional angle of the C140G-N141W bond between the P6 and P5 residues with the TORSION facility QUANTA (see Methods). The results of 10° incremental changes to from -90° to 90° showed that the overlap was not diminished unless rotated so as to become 'buried' in the 'substrate' itself. This creates an unacceptable

conformation of the 'substrate' and shows that rigid body movement of the autolysis loop cannot alleviate the overlap between the two structures. This finding, in fact, became the reason for utilizing molecular mechanics minimizations to alleviate the steric overlap.

The identification of the 'substrate' P1'-P5' residues as constituting the majority of the overlap in the initial autolysis dimers (which consequently must adapt for productive binding to occur) has several significant implications. First, these residues were shown to have extremely high thermal mobility, which is consistent with their role in adapting to form the Michaelis complex. Second, their mobility is probably important for the successful scission of the peptide bond around 145K-146S. Mobility and a 'non-rigid' conformation of the P1'-P5' residues is probably important for allowing solvent water to enter and act as the nucleophile in the deacylation step of proteolysis. Third, the extent of the motion, in some instances up to 4-5 Å for individual alpha carbons, gives an indication of the extent of backbone movement capable of resulting from an activated process in protein-protein association. These changes are far greater than the averages (and standard deviations) of the 13 'substrate' overlapping residues in the SP results of Table 2.3. The changes of four comparisons there are:

	'Substrate'	P1'-P5'
1tpo-3ptp	0.38 ± 0.43	0.80 ± 0.55
1tpo-3ptb	0.09 ± 0.06	0.08 ± 0.04
1tpo-2ptc	0.46 ± 0.23	0.70 ± 0.19
1tpo-1tpa	0.43 ± 0.23	0.66 ± 0.25

These observations are consistent with the statement of Fontana et al. (1986) that:

> "One may envision the overall process of proteolysis in which the initial interaction of the globular protein with the protease involves recognition of a specific amino acid sequence of that site, after which some local conformational change takes place in order to make the idealized transition state of the cleavage reaction."

Conclusion: Molecular mechanics energy minimizations, performed to remove the steric overlap in the initial configurations of β-trypsin autolysis dimers, found the extent of the 'substrate' autolysis site alpha carbon changes to be greater than 2 Å if the 'enzyme' backbone is constrained in such a manner as to reproduce the crystal changes observed in Chapters 1 and 2. Assuming that the 'conformational change', hypothesized to be induced by calcium, is reflected in the trypsin crystal structures analyzed and that this change is necessary to take on a [nearly] perfect 'lock and key' fit between the 'enzyme' and 'substrate', these changes would be anticipated to be large enough to be detected in the FTIR analyses of Chapter 4. The extent of these changes also sheds light on the magnitude of backbone changes resulting from activated processes in protein-protein association. Future solvated molecular dynamics, with and without calcium, will undoubtedly lead to new insights into these phenomena.

Table 3.1

'Enzyme' Screened Overlapped Atoms

2ptc-1tpo (P2 & P1 + CD equivalence)

screened from -30 -30 -30 to 30 30 30
minimum number of overlapped atoms = 24 and 25

		0	12	12	14	8	8	8	8	10	10
		0	-4	-2	-2	-8	-6	0	2	-8	-6
		0	22	26	24	24	22	20	20	22	22
39Y	CB					x				x	x
40H	O		x	x	x		x	x		x	x
41F	C							x	x		
	O	x	x	x	x	x	x	x		x	x
42C	CA					x		x			
43G	N		x	x	x	x	x	x	x	x	x
55A	CB	x	x	x	x	x	x		x	x	x
97N	CB	x					x				
143N	CB			x	x			x	x		x
151Y	CB		x	x	x			x	x		
173P	O					x					
174G	O						x				
192Q	C	x						x	x		
	CB		x	x	x	x	x	x	x	x	x
193G	N	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x
	O	x				x					
195S	N	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x
196G	N	x	x	x	x	x	x	x	x	x	x
213V	O	x								x	
214S	N	x						x			
	CA	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x
215W	N	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x
	C		x	x	x	x	x			x	x
	O					x	x			x	x
	CB	x	x	x	x	x	x	x	x	x	x
216G	O					x					
219G	CA								x		

Table 3.1 (cont'd)

'Enzyme' Screened Overlapped Atoms

2ptc-1tpo

		10	10	10	10	12	12	12	12	12	14	14
		-6	-4	-2	-2	-4	-2	-2	0	0	-4	-2
		24	24	22	24	24	22	24	22	24	22	22
39Y	CB	x									x	
40H	O	x	x	x	x	x	x	x	x	x	x	x
41F	C											
	O	x	x	x	x	x	x	x	x	x	x	x
42C	CA											x
43G	N		x	x	x	x	x	x	x	x	x	
55A	CB	x	x	x	x	x	x	x	x	x	x	x
97N	CB											
143N	CB	x	x	x	x	x	x	x	x	x	x	x
151Y	CB		x	x	x	x	x	x	x	x	x	x
173P	O											
174G	O											
192Q	C			x	x		x	x	x	x		x
	CB	x	x	x	x	x	x	x	x	x	x	x
193G	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O											
195S	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
196G	N	x	x	x	x	x	x	x	x	x	x	x
213V	O											
214S	N			x								
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
215W	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x			x						
	CB	x	x	x	x	x	x	x	x	x	x	x
216G	O											
219G	CA											

Table 3.1 (cont'd)

'Substrate' Screened Overlapped Atoms

2ptc-1tpo

		0	12	12	14	8	8	8	8	10	10
		0	-4	-2	-2	-8	-6	0	2	-8	-6
		0	22	26	24	24	22	20	20	22	22
17V	CB		x	x	x	x	x	x	x	x	x
19G	O								x		
40H	O	x					x				
74N	CB					x	x				
146S	N	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x
	C	x		x		x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x
147S	N	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	
148G	N	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x
	C	x				x	x	x	x		
	O	x	x	x	x	x	x	x	x	x	x
149T	N	x						x	x	x	
	CA	x				x				x	x
	C	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x
	CB	x									
150S	N	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x
151Y	N		x	x	x	x	x	x	x	x	x
	CA	x				x					
152P	CB					x					
186E	C				x						
	O		x	x	x			x	x		
188G	CA			x	x			x	x		
	C			x	x				x		
	O		x	x	x						x
221Q	CA		x		x					x	
	CB		x	x	x		x	x		x	x
222K	O					x				x	x

Table 3.1 (cont'd)

'Substrate' Screened Overlapped Atoms

2ptc-1tpo

		10 -6 24	10 -4 24	10 -2 22	10 -2 24	12 -4 24	12 -2 22	12 -2 24	12 0 22	12 0 24	14 -4 22	14 -2 22
17V	CB	x	x	x	x	x	x	x	x	x	x	x
19G	O											
40H	O											
74N	CB											
146S	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
147S	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
148G	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C			x	x				x	x		
	O	x	x	x	x	x	x	x	x	x	x	x
149T	N											
	CA	x										
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB											
150S	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
151Y	N	x	x	x	x	x	x	x	x	x	x	x
	CA											
152P	CB											
186E	C											
	O		x	x	x	x	x	x	x	x	x	x
188G	CA			x	x		x	x	x	x		
	C							x	x	x		
	O	x	x	x	x	x	x	x	x	x	x	x
221Q	CA					x					x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
222K	O	x									x	x

Table 3.2

'Enzyme' Screened Overlapped Atoms

2ptc-3ptp (P2 & P1 + CD equivalence)

screened from -30 -30 -30 to 40 30 30
minimum number of overlapped atoms = 29

		0	20	22	22	22	30	30
		0	18	18	18	20	10	16
		0	12	10	12	6	20	12
39Y	CB						x	x
40H	O						x	x
41F	CB		x	x	x	x		x
42C	CA						x	
	C						x	
43G	N		x	x	x		x	x
55A	CB		x	x	x	x	x	x
57H	C	x						
	CB	x						x
151Y	CB							x
191C	C					x		
	O	x	x	x	x	x		x
192Q	N					x		
	CA	x	x	x	x	x		x
	C	x	x	x	x	x	x	x
	O		x	x	x	x		x
	CB	x			x		x	x
193G	N	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x
	O	x	x	x	x	x		
194D	N	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x
195S	N	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x
196G	N	x	x	x	x	x	x	x
	CA		x	x	x	x	x	
214S	CA	x	x	x				
	C	x	x	x	x	x	x	
	O	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	
215W	N	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x

Table 3.2 (cont'd)

'Substrate' Screened Overlapped Atoms

2ptc-3ptp

		0	20	22	22	22	30	30
		0	0	18	18	20	10	16
		0	0	10	12	6	20	12
17V	CA						x	
	C						x	
	CB				x		x	x
18G	N						x	
145K	CD		x	x	x	x		x
146S	CA	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x
147S	N	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x
148G	N	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x
149T	N	x	x	x	x	x		x
	CA	x				x	x	
	C	x	x	x	x		x	x
	O	x	x	x	x	x	x	
	CB	x				x		x
150S	N	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x
	C	x	x	x	x		x	x
	O	x	x	x	x		x	
	CB	x	x	x	x	x	x	x
151Y	N	x					x	
186E	C							x
	O							x
219G	CA		x	x	x	x		x
220C	N							x
221Q	N						x	
	CA						x	x
	CB						x	x
222K	O						x	
224K	CB							x

Table 3.3

'Enzyme' Screened Overlapped Atoms

1tpo-1tpo (P2 & P1 + CD equivalence)

screened from -30 -30 -30 to 30 30 30
minimum number of overlapped atoms = 25

		0	8	8	10	10	12	8	8	10	10	10
		0	-2	0	-2	-2	-2	-4	0	-4	-4	0
		0	22	22	22	24	24	24	24	22	24	22
40H	O				x		x			x	x	
41F	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x										
42C	CA							x	x			
43G	N		x	x	x	x	x	x	x	x	x	x
55A	CB	x	x	x	x	x	x	x	x	x	x	x
57H	O	x										
96S	N	x										
97N	N	x										
	CB	x	x									
99L	CA	x										
143N	CB		x	x	x	x	x	x	x	x	x	x
149T	CB			x					x			x
151Y	N											x
	CB		x	x	x	x	x	x	x	x	x	x
192Q	CA	x										
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x										
	CB		x	x	x	x	x	x	x	x	x	x
193G	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x										
194D	N	x										
	CA	x										
	C	x										
	O	x										
195S	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
196G	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x										
214S	N	x										
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
215W	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O							x		x	x	
	CB	x	x	x	x	x	x	x	x	x	x	x

Table 3.3 (cont'd)

'Substrate' Screened Overlapped Atoms

1tpo-1tpo

		0	8	8	10	10	12	8	8	10	10	10
		0	-2	0	-2	-2	-2	-4	0	-4	-4	0
		0	22	22	22	24	24	24	24	22	24	22
17V	CB		x	x	x	x	x	x	x	x	x	x
40H	O	x	x									
	CB	x										
146S	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x										
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
147S	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
148G	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x			x	x	x		x
	O	x	x	x	x	x	x	x	x	x	x	x
149T	N	x										
	CA	x	x		x	x	x	x		x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x										
150S	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x	x	x	x	x	x	x	x	x	x	x
	C	x	x	x	x	x	x	x	x	x	x	x
	O	x	x	x	x	x	x	x	x	x	x	x
	CB	x	x	x	x	x	x	x	x	x	x	x
151Y	N	x	x	x	x	x	x	x	x	x	x	x
	CA	x										
	CB	x										
184Y	CB			x					x			x
186E	O		x	x	x	x	x	x	x	x	x	x
188G	CA		x	x	x	x	x		x			x
	C			x	x	x	x		x		x	x
	O		x	x	x	x	x	x	x	x	x	x
192Q	CB	x										
219G	CA	x										
221Q	CB				x		x			x	x	

Table 3.4

'Enzyme' Screened Overlapped Atoms

1tpo-3ptp (P2 & P1 + CD equivalence)

screened from -30 -30 -30 to 30 30 30
minimum number of overlapped atoms = 31

		0	18	24	26	26	26
		0	12	14	4	4	6
		0	14	16	14	16	16
39Y	CB				x	x	x
40H	O				x	x	x
43G	N		x	x	x	x	x
57H	N	x					
	CA	x					
	C	x		x			
	CB	x					
95N	CA	x					
	C	x					
	O	x					
96S	N	x					
	CA	x					
	C	x					
	O	x					
	CB	x					
97N	N	x	x				
	CA	x					
	O	x					
	CB	x					
98L	N	x					
	CA	x					
	CB	x					
99L	CB	x					
151Y	CB			x			
173P	O	x					
174G	N	x					
	CA	x					
	C	x					
	O	x					
175Q	N	x					
	CA	x					
	C	x					
	CB	x					
191C	O			x			
192Q	CA	x	x	x	x	x	x
	C	x	x	x	x	x	x
	O	x	x	x	x	x	x
	CB	x		x	x	x	x

Table 3.4 (cont'd)

'Enzyme' Screened Overlapped Atoms

1tpo-3ptp

		0	18	24	26	26	26
		0	12	14	4	4	6
		0	14	16	14	16	16
193G	N	x	x	x	x	x	x
	CA	x	x	x	x	x	x
	C	x	x	x	x	x	x
	O	x	x	x			
194D	N	x	x	x	x	x	x
	CA	x	x	x	x	x	x
	C	x	x	x	x	x	x
	O	x	x	x	x	x	x
	CB	x					
195S	N	x	x	x	x	x	x
	CA	x	x	x	x	x	x
	C	x	x	x	x	x	x
	O	x	x	x	x	x	x
	CB	x	x	x	x	x	x
196G	N	x	x	x	x	x	x
	CA		x	x			
213V	CA	x					
	C	x					
	O	x	x		x	x	x
	CB	x					
214S	N	x	x				
	CA	x	x		x	x	x
	C	x	x	x	x	x	x
	O	x	x	x	x	x	x
	CB	x	x	x	x	x	x
215W	N	x	x	x	x	x	x
	CA	x	x	x	x	x	x
	C	x	x	x	x	x	x
	O	x	x		x	x	x
	CB	x	x	x	x	x	x
216G	N				x	x	x
227V	O	x					

Table 3.4 (cont'd)

'Substrate' Screened Overlapped Atoms

				1tpo-3ptp			
		0	18	24	26	26	26
		0	12	14	4	4	6
		0	14	16	14	16	16
17V	N			x	x	x	x
40H	N		x				
	C	x					
	O	x	x				
	CB	x					
41F	N	x					
	CA	x					
	C	x					
	O	x					
	CB	x					
42C	O	x					
72N	CB	x					
73I	C	x					
	O	x					
74N	N	x					
	CA	x					
	C	x					
	O	x					
	CB	x					
75V	N	x					
	CA	x					
	CB	x					
141W	CB	x					
145K	O	x					
146S	N	x	x	x	x	x	x
	CA	x	x	x	x	x	x
	C	x	x	x	x	x	x
	O	x	x	x	x	x	x
	CB	x	x	x	x	x	x
147S	N	x	x	x	x	x	x
	CA	x	x	x	x	x	x
	C	x	x	x	x	x	x
	O	x	x	x	x	x	x
	CB	x	x	x	x	x	x
148G	N	x	x	x	x	x	x
	CA	x	x	x	x	x	x
	C	x	x	x	x		x
	O	x	x	x	x	x	x
149T	N	x	x		x	x	x
	CA	x	x	x	x	x	x
	C	x	x	x	x	x	x
	O	x	x	x	x	x	x
	CB	x		x			

Table 3.4 (cont'd)

'Substrate' Screened Overlapped Atoms

1tpo-3ptp

		0 0 0	18 12 14	24 14 16	26 4 14	26 4 16	26 6 16
150S	N	x	x	x	x	x	x
	CA	x	x	x	x	x	x
	C	x	x	x	x	x	x
	O	x	x	x			x
	CB	x	x	x	x	x	x
151Y	N	x	x	x	x	x	x
	CA	x					
	C	x					
	CB	x					
153D	CA	x					
	CB	x					
186E	O			x			
192Q	CA	x					
	CB	x					
193G	N	x					
	CA	x					
	C	x					
	O	x					
221Q	CA				x	x	x
	CB				x	x	x
222K	O				x	x	x

Table 3.5

Average CHARMM Final Minimization Energies of the 2ptc-1tpo Autolysis Dimers

Constraints	Energy (kcal/mol)
NONE	- 13641 ± 1568
NONE + CALCIUMS	- 14347 ± 1700
N	- 15305 ± 1682
N + CALCIUMS	- 15806 ± 1738
N, C	- 14197 ± 2115
N, C + CALCIUMS	- 14517 ± 1799
BACKBONE	- 12939 ± 3437
BACKBONE + CALCIUMS	- 13107 ± 2059

Table 3.6

Average CHARMM Final Minimization Energies
of the 2ptc-3ptp Autolysis Dimers

Constraints	Energy (kcal/mol)
NONE	- 14108 ± 1653
NONE + CALCIUMS	- 14832 ± 2129
N	- 15225 ± 1646
N + CALCIUMS	- 15736 ± 1752
N, C	- 13868 ± 1931
N, C + CALCIUMS	- 14681 ± 1966
BACKBONE	- 10289 ± 6395
BACKBONE + CALCIUMS	- 14383 ± 1802

Table 3.7

Average CHARMM Final Minimization Energies of the 1tpo-1tpo Autolysis Dimers

Constraints	Energy (kcal/mol)
NONE	- 15439 ± 995
NONE + CALCIUMS	- 16042 ± 974
N	- 16649 ± 1034
N + CALCIUMS	- 17102 ± 914
N, C	- 15258 ± 948
N, C + CALCIUMS	- 16065 ± 951
BACKBONE	- 13883 ± 1568
BACKBONE + CALCIUMS	- 14447 ± 1207

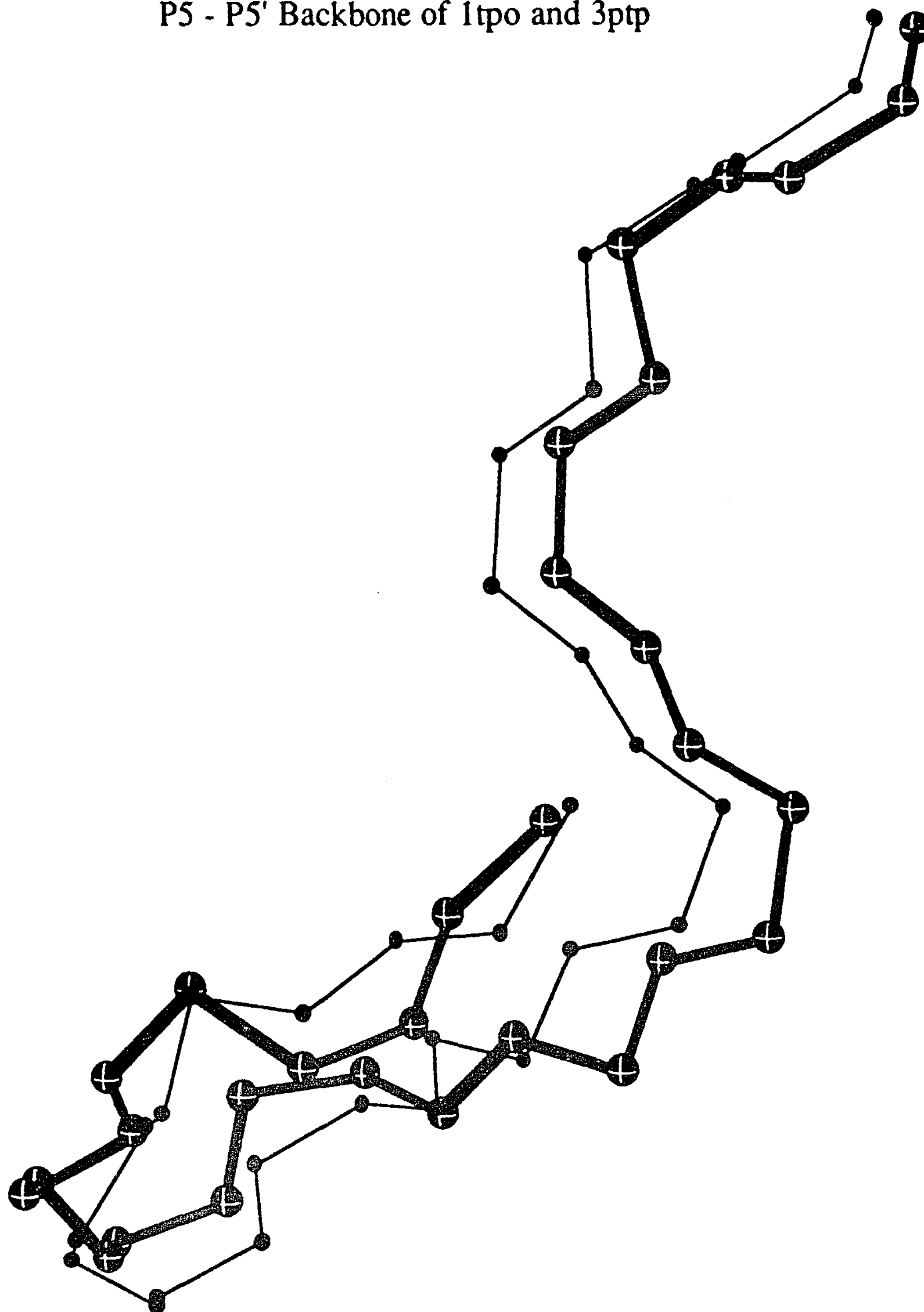
Table 3.8

Average CHARMM Final Minimization Energies of the 1tpo-3ptp Autolysis Dimers

Constraints	Energy (kcal/mol)
NONE	- 15620 ± 230
NONE + CALCIUMS	- 16087 ± 388
N	- 16428 ± 3010
N + CALCIUMS	- 17115 ± 619
N, C	- 15170 ± 1053
N, C + CALCIUMS	- 15600 ± 1103
BACKBONE	- 5030 ± 3963
BACKBONE + CALCIUMS	- 5331 ± 4476

Figure 3.1

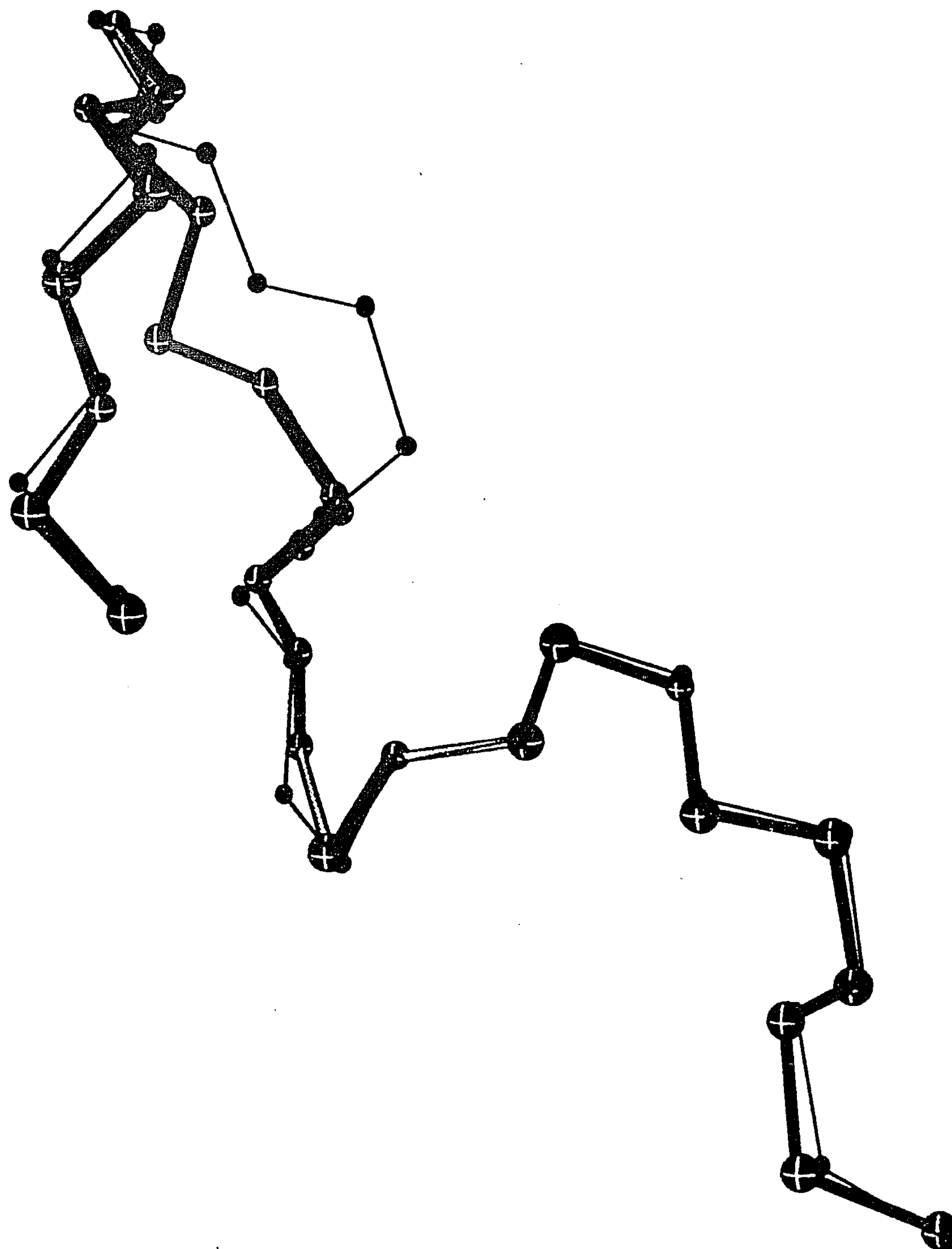
P5 - P5' Backbone of 1tpo and 3ptp



1tpo = ball and stick figure
3ptp = thicker bonds and larger atoms

Figure 3.2

Initial and Final P5 - P5' Backbone Positions of 2ptc-1tpo Autolysis Dimer



Initial Conformation = ball and stick figure
Final Conformation = thicker bonds and larger atoms

Chapter 4

Infrared Spectroscopic Studies of Calcium Binding to Inhibited β-Trypsins

Goal

To study the effect of calcium binding on two inhibited forms of bovine β-trypsin by Fourier-transform infrared spectroscopy (FTIR) with particular emphasis on its effect on the absorption of the band at 1654 cm^{-1}.

4.1 Results

Figure 4.1 shows the ion exchange chromatography elution pattern for separation of β- and α-trypsin as described in Methods. Figures 4.2 and 4.3 show the curve-fitted spectra of BA-trypsin, with and without calcium. Figures 4.4 and 4.5 show the curve-fitted spectra of DIP-trypsin, with and without calcium.

Tables 4.1 and 4.2 list the peak positions, normalized half-heights, half-widths, and relative band areas of the amide I' component bands of BA- and DIP-inhibited forms of bovine β-trypsin in the presence and absence of 20 mM calcium respectively. The largest observed difference in the relative intensities upon calcium binding is 0.02 in the 1633 cm^{-1} band of BA-trypsin. This value, however, is accentuated due to roundoff error and is actually 0.012 (0.236 - 0.224), which is approximately equal to the variability observed in the 1625 - 1690 cm^{-1} spectra of equivalent samples (Prestrelski et al., 1991). Thus, these results indicate that calcium binding has no effect on the secondary structure of both the DIP- and BA-inhibited forms of β-trypsin detectable by FTIR spectroscopy. In addition, a new, weak, band appears at 1699 cm^{-1} in the spectra of BA-trypsin.

4.2 Discussion

Although trypsin has been one of the most extensively studied enzymes of this century, to date, there has been no conclusive evidence as to calcium's mechanism of action in slowing trypsin autolysis. Investigators have proposed various mechanisms (see Chapter 6), with the majority of these hypothesizing that calcium exerts its action via induction of a conformational change in the autolysis loop of β-trypsin.

The band assignments for these studies are essentially the same as those noted by Prestrelski et al. (1991). In that study, the autolysis loop of native bovine β-trypsin was shown to contribute to absorption at 1654 cm^{-1}. Alpha helices of polypeptide chains have previously been shown to absorb here (Byler & Susi, 1986; Surewicz & Mantsch, 1988). The absence of observable change in the integrated intensities of any of the amide I' bands, and especially the 1654 cm^{-1} band, in the two inhibited β-trypsin forms, thus supports the more specific conclusion that calcium binding does not perturb the backbone conformation of the autolysis loop of β-trypsin.

At present it is not clear why the observed integrated relative intensity of the 1654 cm^{-1} band is lower in BA-trypsin than it is in the native species (Prestrelski et al., 1991). Distinct changes in the atomic positions of the autolysis loop backbone of DIP-trypsin relative to the native structure were previously found and correlated to absorbance at 1654 cm^{-1} (Prestrelski et al., 1991) but the reported x-ray structure of bovine BA-trypsin is virtually identical to that of native trypsin as shown by Liebman (1986) and in the SP results of Chapter 2. Several possible explanations may be put forward for this apparent incongruity. The increase in absorbance at 1643 cm^{-1} could possibly be due to partial autolysis having occurred during storage at - 10° C (Schroeder & Shaw, 1968). If partial autolysis did not occur, this result could be due to the fact that infrared spectroscopy in the amide I' region is more sensitive than x-ray diffraction to very small changes ($\leq$ 0.1 Å) in the geometry of a hydrogen bonding environment. The autolysis loop may absorb near 1643 cm^{-1} in the two active site inhibited forms but at 1654 cm^{-1} in the native form.

Finally, it could be due to the fact that the solution conformation of BA-trypsin is different than that in the crystalline state. Nevertheless, the conclusion concerning the effect of calcium binding on the conformation of β-trypsin is unaffected by this incongruity.

The weak band present at 1699 cm^{-1} in the spectrum of BA-trypsin is not seen in the spectrum of either DIP- or native β-trypsin (Prestrelski et al., 1991). This feature is thus, most likely, not an amide I' component, but instead may be attributed to the antisymmetric NCN stretching vibration of the benzamidinium ion hydrogen bonded to an active site aspartate group of β-trypsin. This interpretation is consistent with the structural details reported for this complex in the x-ray diffraction study of BA-trypsin (Bode & Schwager, 1975). In addition, in simple salts formed by primary amidines with HCl, the antisymmetric NCN stretching mode of the amidinium ion absorbs very strongly between 1685 - 1700 cm^{-1} (Keller, 1986; Mecke & Kutzelnigg, 1960). The intensity of this band in the simple salts is so intense that it is reasonable to assume that the analogous band would be visible as a weak feature in BA-trypsin because there is only one bound benzamidine group for every 223 backbone carbonyls of bovine trypsin. In fact, the relative integrated intensity of 0.007 is in close proportion to the total number of absorbing carbonyl and amidinium groups in the enzyme and inhibitor.

Conclusion: Fourier-transform infrared spectroscopy was used to examine the effect of calcium binding on the secondary structure of two inhibited bovine β-trypsins. Neither the diisopropylfluorophosphate- nor benzamidine-inhibited forms showed detectable secondary structure perturbation upon calcium binding at pD 6.9 and 5.0, respectively. Considered in light of the recent assignment of an amide I' band to the autolysis loop of bovine β-trypsin, these results contradict the generally-held hypothesis that calcium slows trypsin autolysis by induction of a conformational change at this site and support the contention that the mechanism of action has a specific electrostatic origin. In addition, the appearance of a band at 1699 cm^{-1} in the BA-trypsin can be interpreted as resulting from the C-N stretching vibrations of the amidinium moiety, which the observed crystal structure indicates is hydrogen-bonded to the carboxyl group of active-site 189D.

Table 4.1

Peak Positions, Half-Heights, Half-Widths, and Relative Integrated Intensities[a] of BA-Inhibited Trypsin

	NO CALCIUM			20 mM CALCIUM		
ν (cm^{-1})	HH	HW	RII	HH	HW	RII
1625	0.3	2.5	.06	0.3	2.5	.06
1633	1.0	3.0	.24	0.9	3.2	.22
1643	0.8	5.0	.31	0.8	4.9	.31
1654	0.5	2.7	.11	0.5	2.7	.11
1663	0.5	3.0	.12	0.6	2.8	.13
1673	0.4	2.4	.07	0.4	2.4	.07
1683	0.4	2.2	.07	0.4	2.3	.07
1693	0.2	1.7	.02	0.2	1.8	.02
1699	0.1	1.3	.007	0.1	1.5	.007

[a] The relative intensities at all peak positions, except 1699 cm^{-1}, were rounded off to the nearest hundredths and thus may not sum to 1.00

HH = half-height (normalized to total areas)
HW = half-width
RII = relative integrated intensity

Table 4.2

Peak Positions, Half-Heights, Half-Widths, and Relative Integrated Intensities[a] of DIP-Inhibited Trypsin

	NO CALCIUM			20 mM CALCIUM		
ν (cm^{-1})	HH	HW	RII	HH	HW	RII
1625	0.5	2.9	.08	0.5	2.9	.08
1633	1.3	3.4	.25	1.2	3.3	.24
1643	0.9	5.4	.28	0.9	5.8	.29
1654	0.6	2.9	.11	0.6	2.9	.10
1663	0.6	3.4	.12	0.6	3.4	.13
1673	0.4	2.6	.07	0.4	2.7	.07
1683	0.4	2.7	.06	0.3	3.0	.06
1693	0.3	2.1	.03	0.3	2.2	.04

[a] The relative intensities at all peak positions were rounded off to the nearest hundredths and thus may not sum to 1.00

HH = half-height (normalized to total areas)
HW = half-width
RII = relative integrated intensity

Figure 4.1

Ion Exchange Chromotography of Bovine Trypsin

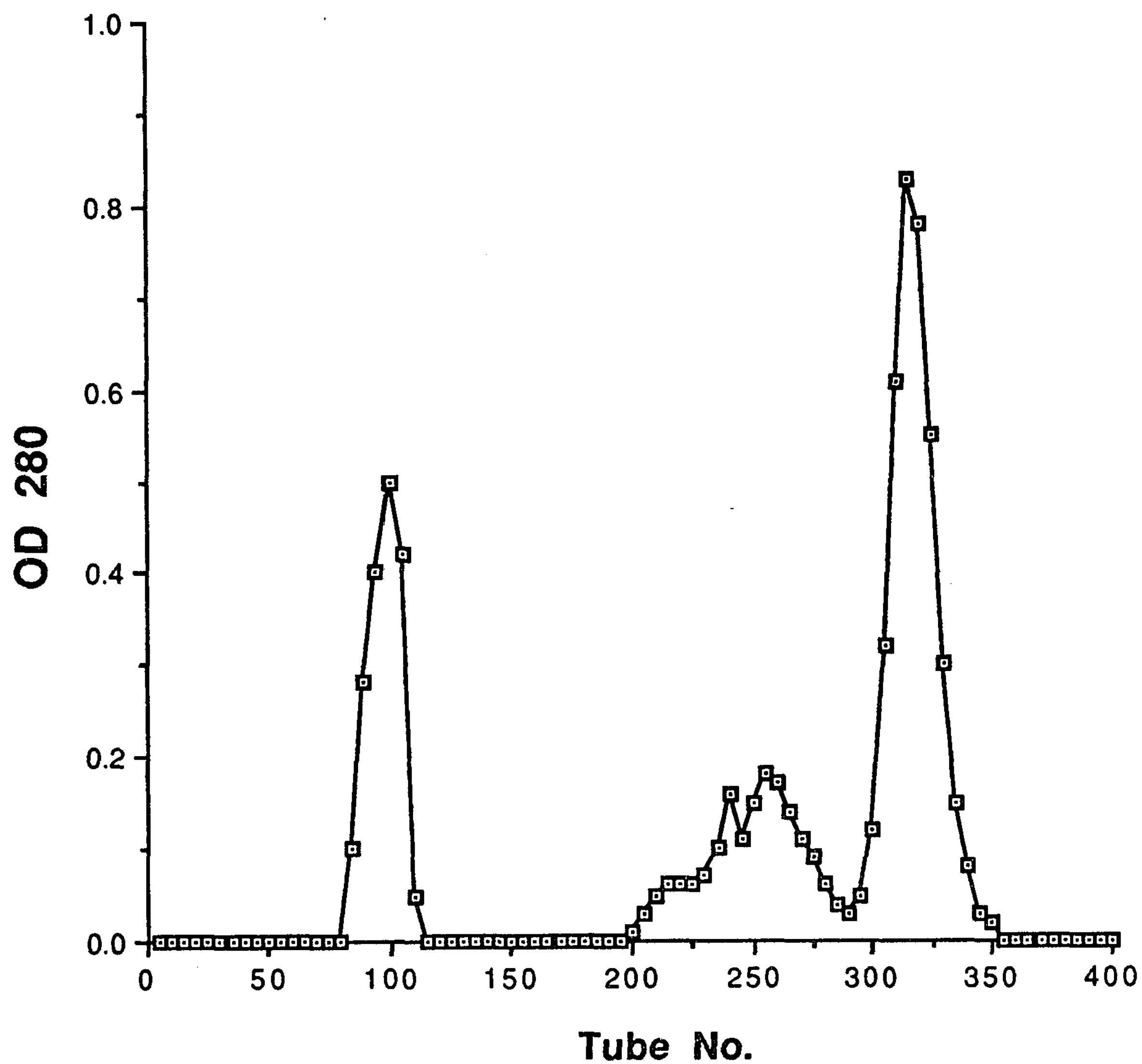


Figure 4.2

Curve-Fitted Spectra of BA-Trypsin (NO CALCIUM)

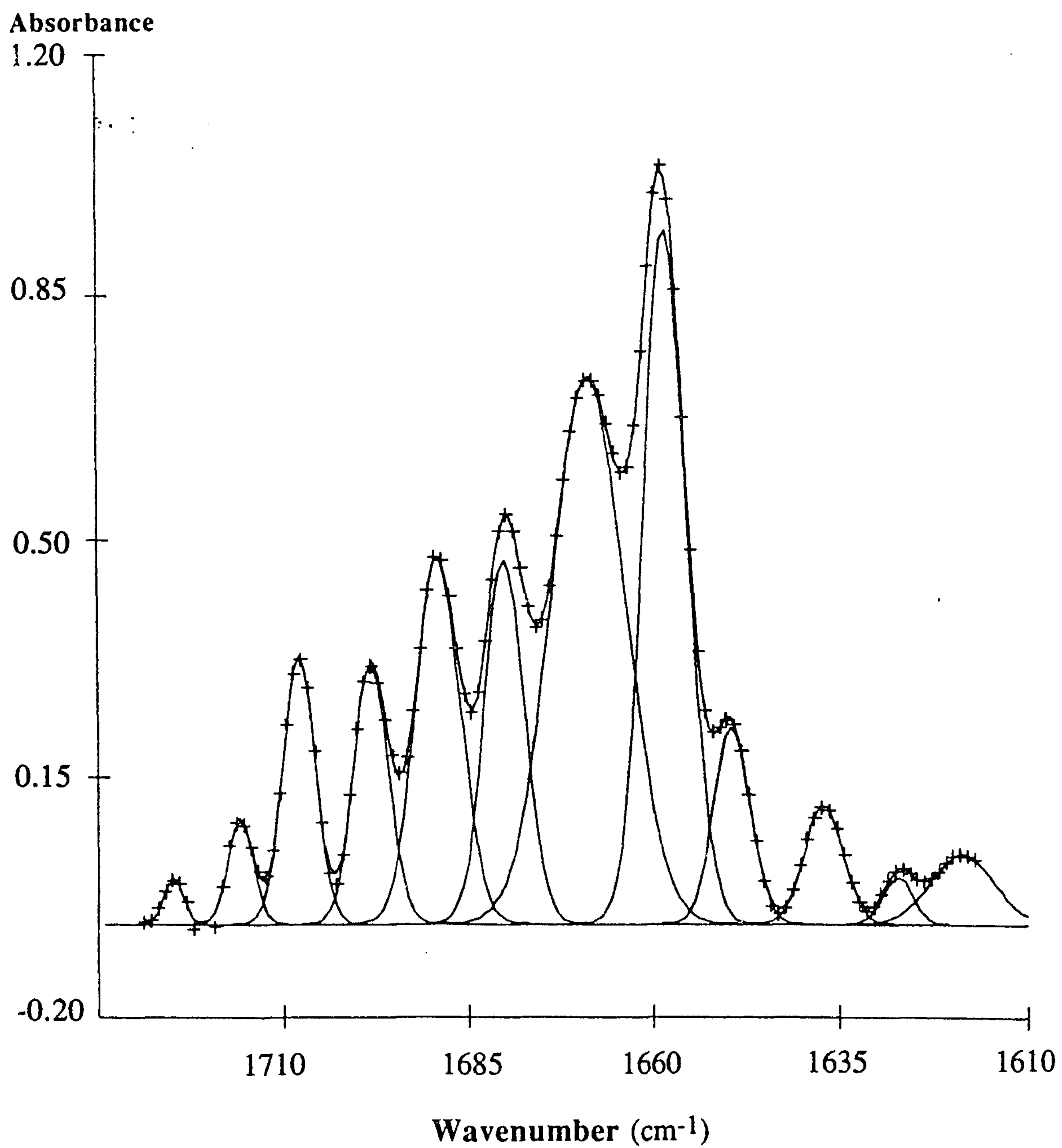


Figure 4.3
Curve-Fitted Spectra of BA-Trypsin (20mM CaCl2)

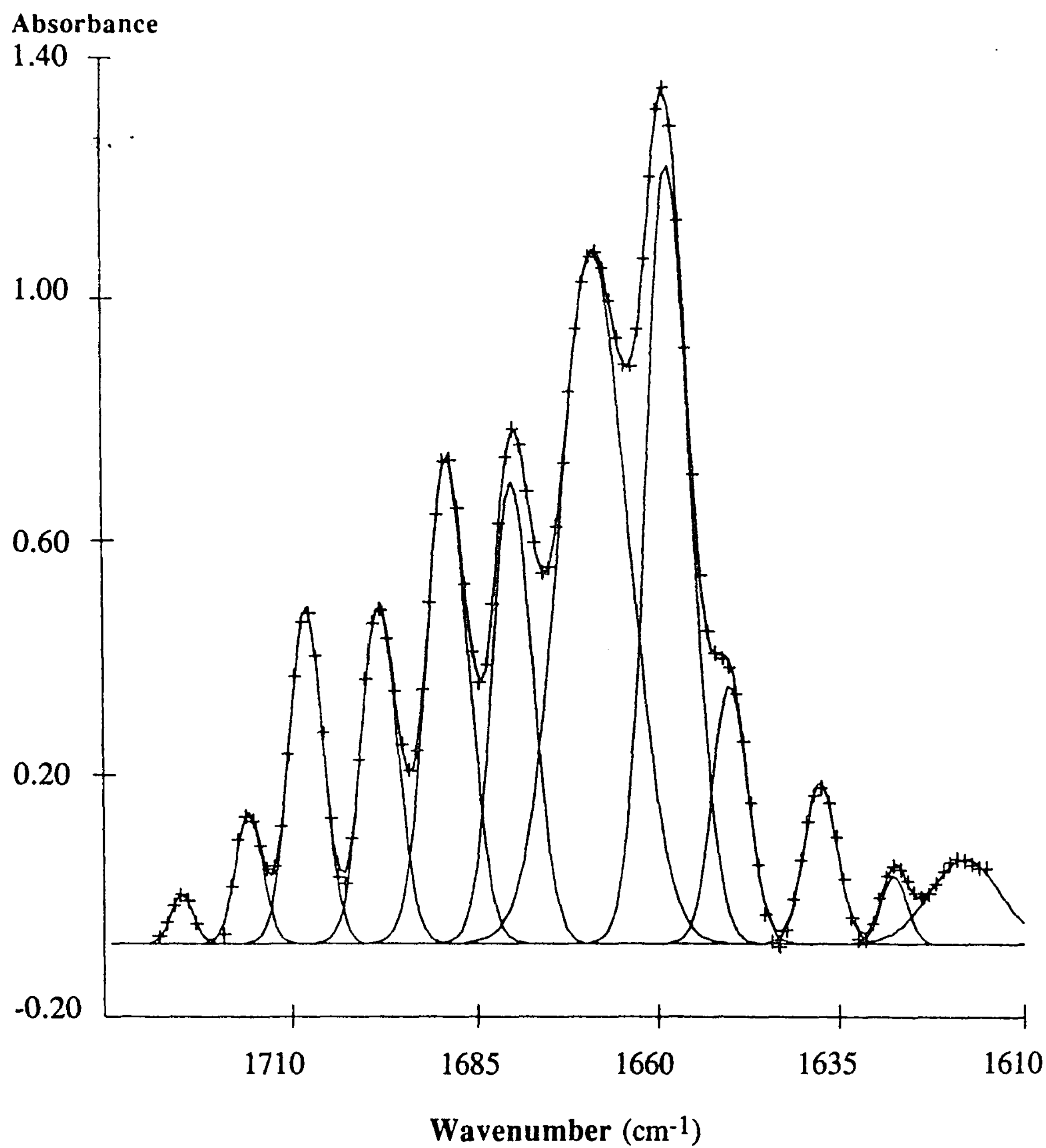


Figure 4.4

Curve-Fitted Spectra of DIP-Trypsin (NO CALCIUM)

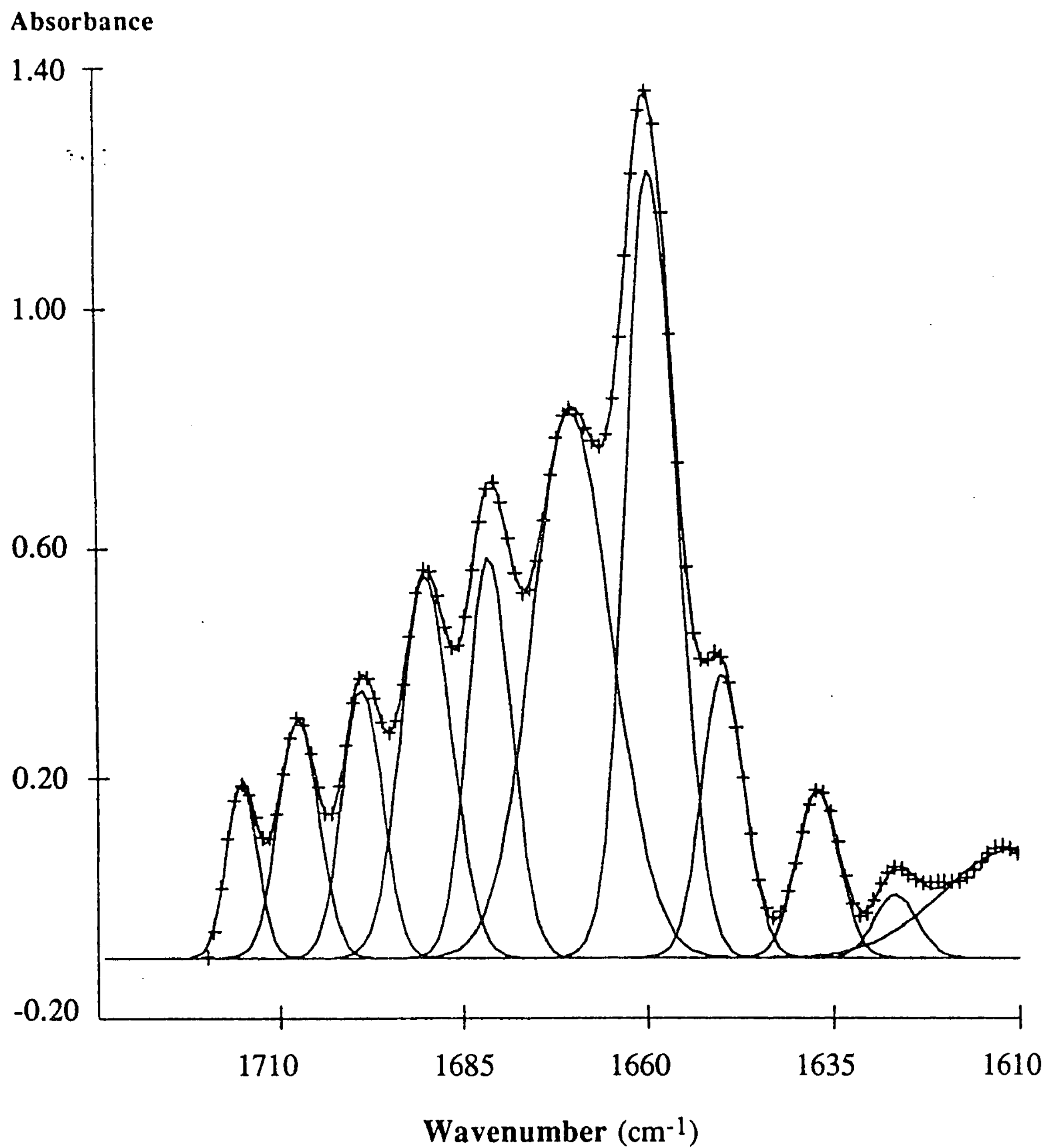
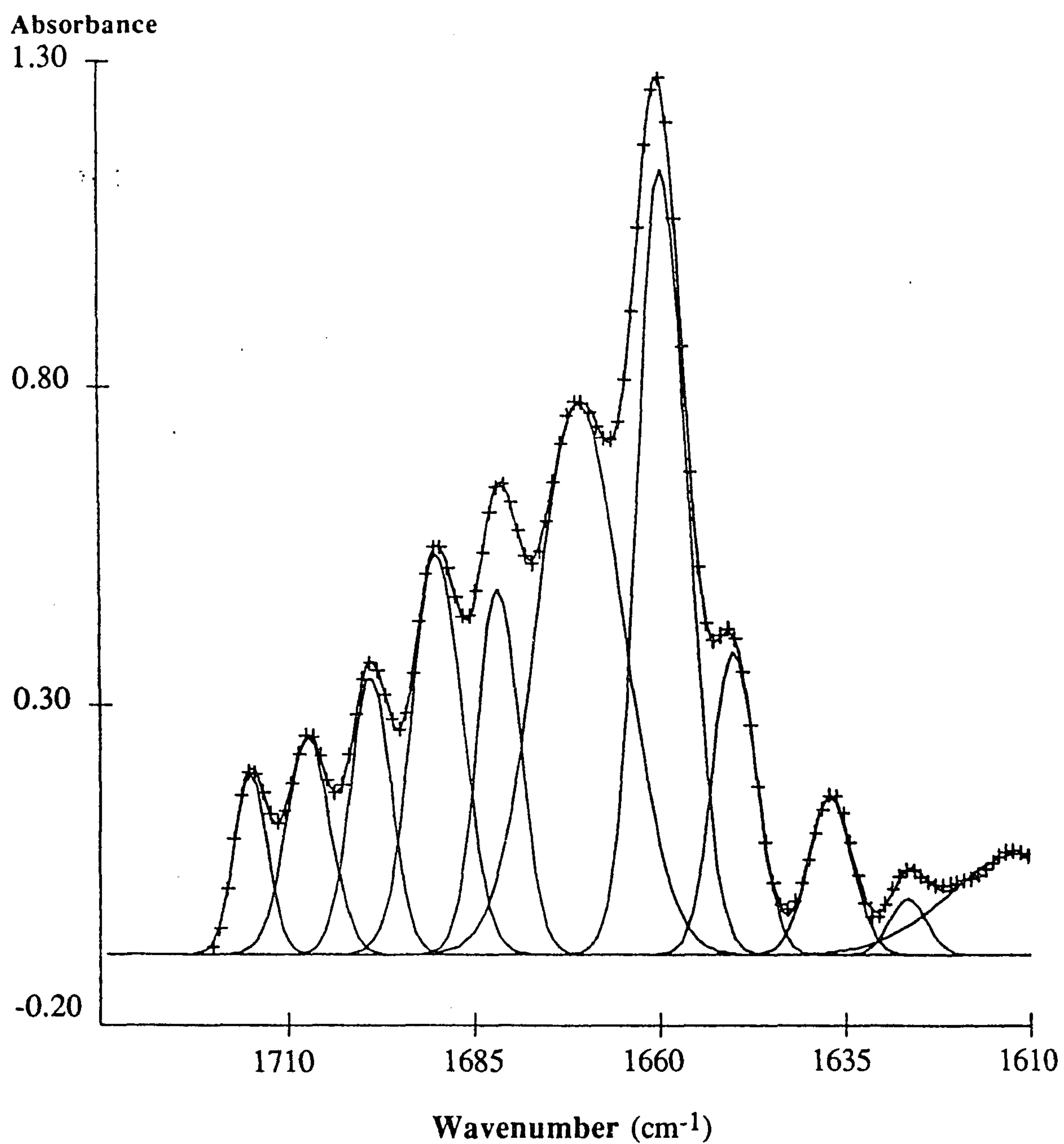


Figure 4.5

Curve-Fitted Spectra of DIP-Trypsin (20mM CaCl2)



Chapter 5
Trypsin Basic Site Analyses

Goal

To explain the specificity of β-trypsin autolysis for the 145K site by examination of the B values, solvent accessibilities, and steric overlap resulting from modeling of the autolysis dimers involving its 16 basic sites.

5.1 Results

Table 5.1 lists the individual and average solvent accessibilities of the P5-P5' residues of all 16 basic sites of the 1tpo crystal structure. All are shown to be at least 20% exposed to solvent. 222K (72%) and 109K (62%) are the two most exposed. The solvent accessibility of 145K is 51%, which ranks third. The least exposed are 107K and 156K (one of the minor cleavage sites of α-trypsin). The 117R and 188K sites have the highest average P5-P1 solvent accessibilities while the 107K and 230K have the lowest. The 145K and 169K have the highest average P1'-P5' solvent accessibilities while the 66R and 204K have the lowest. Furthermore, the 145K site is the only one in which all five of its P1'-P5' residues are more than 40% exposed to solvent. The average solvent accessibilities of all ten amino acids of the basic site P5-P5' residues exhibit a narrower range. The 169K and 117R sites have the highest average values while the 159K and 230K sites have the lowest.

Table 5.2 lists the individual and average P5-P5' B values of the 16 basic sites of 1tpo. The 117R (another minor α-trypsin cleavage site) and 109K alpha carbons have the highest thermal mobilities. The B value of the 145K alpha carbon is consistent with its solvent accessibility - it again ranks third. The least mobile residue is 230K, which is also the fifth most buried of the 16 residues. 188K, the major cleavage site of α-trypsin autolysis to form γ-trypsin, has the fourth highest individual B value of the group. The

117R and 145K sites have the highest average P5-P5' B values, while the 224K and 230K sites having the lowest. The 117R site also has the highest average P5-P1 B value, followed by the 188K site. The 230K site is shown to have the lowest while the 145K site ranks seventh. The 145K site has an average P1'-P5' B value considerably higher than any other site while the 224K site has the lowest. Several sites have similar average P1'-P5' B values, including 109K, 169K, 60K, 107K, and 239K.

Rotational screening results of the 16 basic sites of 'cropped' trypsins as potential substrates of autolysis are shown in Table 5.3. The 145K site is shown to to have the least initial overlap resulting from the P2&P1+CD equivalence. Computational screening found residue 222K to result in the least 'enzyme' overlap, with 21 atoms of the 'enzyme' (30 atoms of the 'substrate') overlapped. Screening of the 145K site results in the second least overlap with 24 atoms of the 'enzyme' (25 atoms of the 'substrate') found to overlap. The 188K α-trypsin cleavage site results in 133 atoms of the 'enzyme' (134 atoms of the 'substrate') overlapped. The two minor cleavage sites of α-trypsin, 156K and 117R, are found to have 164 'enzyme' (171 'substrate') and 118 'enzyme' (118 'substrate)' atoms overlapped respectively. The sites which result in the most steric overlap, as revealed by the rotational screening method, are 66R and 230K.

5.2 Discussion

Rotational screening, B value, and solvent accessibility analyses were employed in an attempt to explain the specificity for the 145K site in the processing of β trypsin to α trypsin. 16 of the 223 amino acids of trypsin are basic and distributed homogeneously over the surface of the enzyme except for the active site cleft. Basic amino acids, the substrates for trypsin, would be repulsed by a positively charge active site cleft. Figures 5.1 and 5.2 (which is rotated 90° about x) highlight the distribution of these 16 residues, along with 195S and the calcium ion, with van der Waals surfaces in the 1tpo structure.

The fact that trypsin is preferentially cleaved at 145K should be borne out in some, or all, of the basic site attributes of 1tpo as listed in Tables 5.1, 5.2, and/or 5.3. This is, of course, based on the assumption that this crystal structure is similar to that found in solution, thus making it a suitable 'substrate'.

The 145K site is significantly more thermally mobile than the other sites and is the only one in which the P1'-P5' residues are all more than 40% exposed to solvent. This is consistent with findings in several other systems. Fontana et al. (1986) found in subtilisin degradation and thermolysin autolysis that "The correlation between sites of highest mobility and sites of limited proteolysis appears to be quite striking." They found that autolysis occurs at two sites between the two structural domains of the enzyme at a "high flexibility peptide loop". Similar findings were made by Porter (1959), where the hinge peptide of immunoglobulin was found to be highly susceptible to proteolysis, and by Richards and Vithayathil (1959), where a mobile region of ribonuclease was found to be susceptible. Furthermore, surface regions with segmental mobility have been found to be antigenic determinants in tobacco mosaic virus, myoglobin, lysozyme, and myohemerythrin (Westhoff et al., 1984; Tainer et al., 1984). Fontana et al. (1986) also concluded that "flexibility of the polypeptide chain of a globular protein at the site of proteolytic attack promotes optimal binding and proper interaction with the active site of the protease." This is apparently the condition found in the autolysis site of β-trypsin.

Analyses of the other 15 basic sites of bovine trypsin leads to an explanation of the specificity for the 145K site. 12 of these sites are readily eliminated by demonstration of excessive steric overlap resulting from modeling of the autolysis dimer with the respective site representing the P1 residue. Only rotational screening of the 60K, 169K, 222K and 239K sites results in less, or just slightly greater, overlap than that found in the 145K site. These sites are found, however, to possess some P1'-P5' residues which are buried and have decreased thermal mobility. The unreactivity of these sites could be explained by their low mobility if mobility of the P1'-P5' residues is important for allowing bulk water to

enter the active site for the deacylation step of peptide hydrolysis. Bartunik (1989) previously hypothesized that mobility of the autolysis site residues might be important for cleavage at this site.

Below is listed a synopsis of the conclusions drawn for each site, other than 145K, as substrates for β-trypsin autolysis:

188K 133 and 144 indicate excessive overlap which cannot be accommodated and adapted.

156K this site is inaccessible, with the lysine being almost totally buried, and has excessive overlap of 164 and 171 atoms.

117R this site is the only one in which the P5-P1 residues are more than 40% exposed, but it has excessive overlap of 118 and 118 atoms.

60K 33 and 40 indicate overlap only slightly higher than the 145K site but it is different in that its P3'-P5' residues are buried.

66R this site is both inaccessible and has excessive overlap.

87K 75 and 77 indicate excessive overlap, about 2 to 3 times greater than that found at the 145K site.

107K the P1 and adjacent residues of this site are inaccessible and it has excessive overlap.

109K 68 and 95 indicate excessive overlap.

159K this site is both inaccessible and moderately overlapped.

169K this site is overlapped 1/3 more than the 145K site and its P2' and P3' residues are buried.

204K 120 and 114 indicate excessive overlap.

222K this site is similar to the 145K site in both its accessibility and overlap, although the thermal mobility of its P1'-P5' site is fifth lowest of the 16 sites and is considerably below that of the 145K site because of buried P3'-P5' residues. Elimination of this site is possible by analogy to PTI, which also has a

basic residue at the P2' position. The P2' residue of this site (224K) was found to overlap with the 'enzyme' in the final orientation and interaction of this residue with the S2' subsite of the enzyme could prohibit bulk water from entering in the deacylation step of the hydrolysis of the peptide.

224K 102 and 115 indicate excessive overlap.

230K this site is both inaccessible and has excessive overlap.

239K this site also has attributes similar to those of the 145K site. The steric overlap is less, but its P2 residue is completely buried and its P2' and P3' residues are more buried than those of the 145K site. This could account for the unreactivity of this site.

Moreover, the analyses also suggests that the conformation of the 188K site changes upon cleavage at the 145K site. The solvent accessibility and average B value of its P1'-P5' residues are low and there is considerable vdW overlap in the autolysis dimer modeled at the 188K site.

Conclusions: The physiological significance of the high thermal variability (and solvent accessibility) of the P1'-P5' residues of the 145K site of bovine β-trypsin is consistent with the findings in other systems that sites of high mobility are most susceptible to proteolysis and immune response. In conjunction with rotational screening used to determine steric overlap, these results indicate that the 145K site is the preferred cleavage site of β-trypsin autolysis. An accurate determination of the electrostatic potential around the basic sites of trypsin, with and without calcium bound, could shed additional light on this question. A conformational change upon cleavage of β-trypsin, necessary for subsequent processing, is also suggested by the excessive overlap found by the screening method at this site.

Table 5.1

Solvent Accessibilities of Basic Sites of 1tpo

Site	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'
145K	2	0	2	12	51	41	72	66	71	42
188K	34	84	14	52	42	4	5	8	54	20
156K	14	39	31	5	20	20	2	1	46	0
117R	64	37	50	73	47	2	22	38	10	43
60K	3	35	5	52	36	74	72	6	26	0
66R	72	6	26	0	22	2	11	10	11	17
87K	13	34	18	51	32	29	22	31	12	69
107K	0	0	0	0	20	1	62	52	48	7
109K	5	0	0	1	62	52	48	7	64	37
159K	5	20	2	1	46	0	27	6	17	36
169K	57	62	19	1	39	63	12	1	60	99
204K	9	4	79	53	34	0	6	0	0	4
222K	59	6	8	40	72	44	31	4	1	0
224K	8	40	72	44	31	4	1	0	1	1
230K	1	0	1	1	30	0	15	43	42	17
239K	17	65	18	1	49	62	10	23	68	56

Average Solvent Accessibilities

Site	P5-P1	P1'-P5'	P5-P5'
145K	13 ± 22	58 ± 16	36 ± 30
188K	45 ± 26	18 ± 21	32 ± 26
156K	22 ± 14	14 ± 20	18 ± 17
117R	54 ± 14	23 ± 18	39 ± 22
60K	26 ± 21	36 ± 26	31 ± 28
66R	25 ± 28	10 ± 5	18 ± 21
87K	30 ± 15	33 ± 22	31 ± 18
107K	4 ± 9	34 ± 28	19 ± 25
109K	13 ± 28	42 ± 22	27 ± 28
159K	15 ± 19	13 ± 15	16 ± 16
169K	36 ± 26	47 ± 40	41 ± 32
204K	36 ± 31	2 ± 3	19 ± 28
222K	37 ± 30	16 ± 20	27 ± 26
224K	39 ± 23	39 ± 23	20 ± 25
230K	7 ± 13	23 ± 19	15 ± 18
239K	30 ± 26	44 ± 26	37 ± 26

Table 5.2

Individual B Values of the Basic Sites of 1tpo

Site	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'
145K	19	14	13	14	22	24	29	27	22	19
188K	20	22	23	26	20	16	13	16	15	13
156K	17	19	15	15	14	15	11	17	18	15
117R	21	22	31	29	24	17	16	16	16	15
60K	10	15	13	15	18	26	25	22	16	5
66R	25	22	16	15	17	15	18	19	13	13
87K	15	18	15	15	17	17	16	15	14	14
107K	15	13	11	12	16	18	24	19	17	15
109K	11	12	16	18	24	19	17	15	21	22
159K	15	14	15	11	17	18	15	16	16	20
169K	21	21	18	16	19	18	18	14	20	24
204K	12	14	19	17	14	15	13	12	13	12
222K	15	14	15	21	17	19	16	18	10	12
224K	15	21	17	19	16	18	10	12	11	11
230K	10	12	11	11	10	13	16	11	15	16
239K	16	16	15	14	16	17	15	16	21	22

Average B Values of Basic Site P5-P5' Residues

Site	P5-P1	P1'-P5'	P5-P5'
145K	16 ± 4	24 ± 4	20 ± 6
188K	22 ± 3	15 ± 2	18 ± 5
156K	16 ± 2	15 ± 3	16 ± 2
117R	25 ± 4	16 ± 1	21 ± 6
60K	14 ± 3	19 ± 9	17 ± 7
66R	19 ± 4	15 ± 3	17 ± 4
87K	16 ± 1	15 ± 1	16 ± 1
107K	13 ± 2	19 ± 3	16 ± 4
109K	16 ± 5	19 ± 3	18 ± 4
159K	14 ± 2	17 ± 2	16 ± 2
169K	19 ± 2	19 ± 4	19 ± 3
204K	14 ± 2	13 ± 1	15 ± 3
222K	16 ± 3	15 ± 4	16 ± 3
224K	18 ± 3	12 ± 3	15 ± 4
230K	11 ± 1	14 ± 2	13 ± 2
239K	15 ± 1	18 ± 3	17 ± 3

Table 5.3

Trypsin Basic Site Screening Analyses

Site	r (Ca++)	C15K-xxxK	Steric Overlap Initial Enz	Steric Overlap Initial Sub	Steric Overlap Final Enz	Steric Overlap Final Sub
145K	22	0.26	40	34	24	25
188K	28	0.60	425	409	133	144
156K	14	0.19	434	447	164	171
117R	13	0.99	371	346	118	118
60K	22	0.49	331	315	33	40
66R	9	0.05	457	487	196	199
87K	24	0.66	387	382	75	77
107K	21	0.79	118	115	103	111
109K	20	0.48	181	181	68	95
159K	23	0.34	91	96	56	51
169K	39	3.32	105	91	40	55
*		0.00	65	62	18	22
204K	25	0.32	296	307	120	104
222K	34	0.89	56	54	21	30
224K	32	0.37	334	340	102	115
230K	29	0.59	276	259	189	197
239K	30	0.71	63	72	17	21

all superpositionings done with P2&P1+CD equivalence
number of atoms in 'cropped ' trypsins = 1091

* = 169K CA translated to PTI position of CA15K

Figure 5.1

Basic Amino Acids of 1tpo

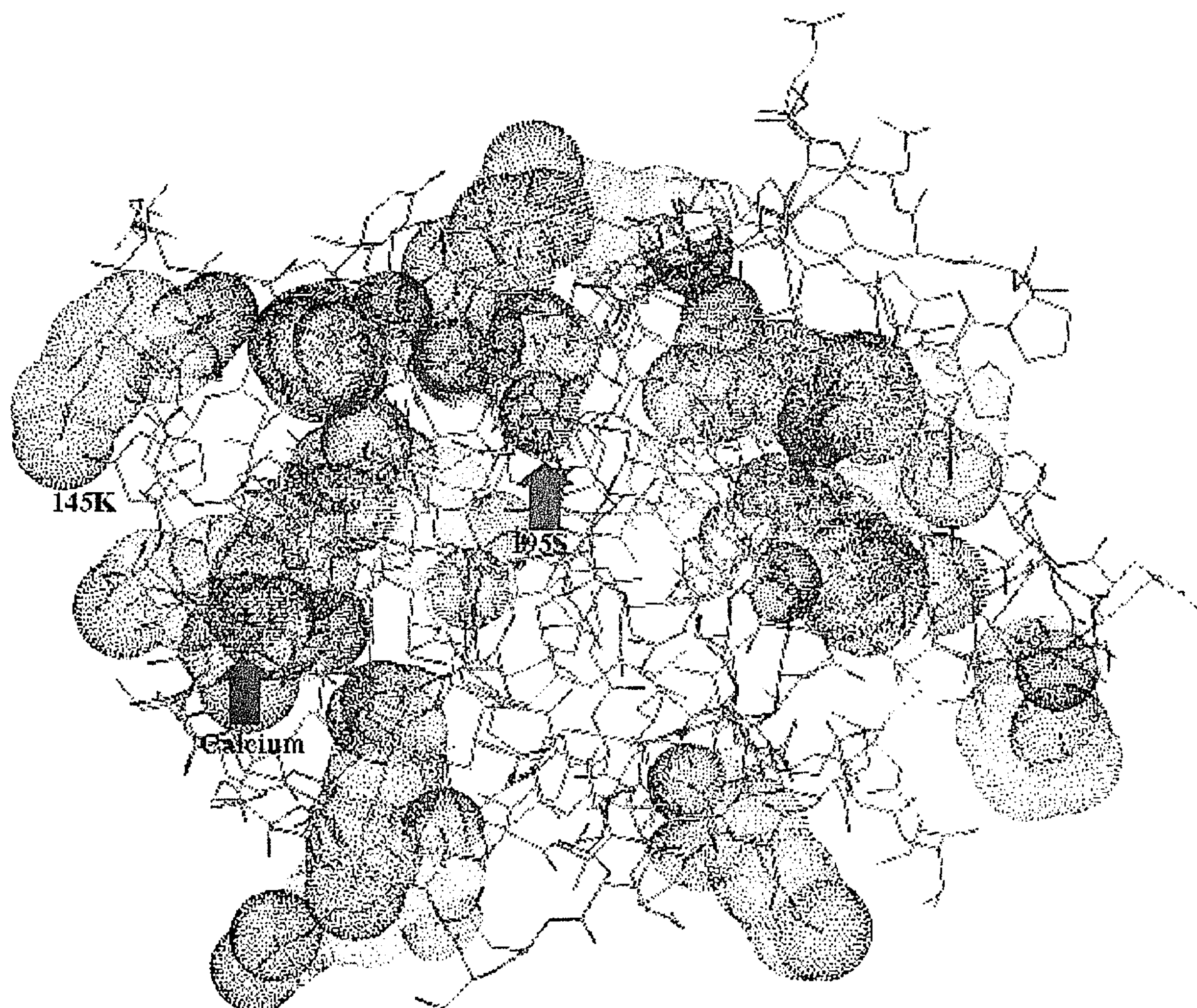
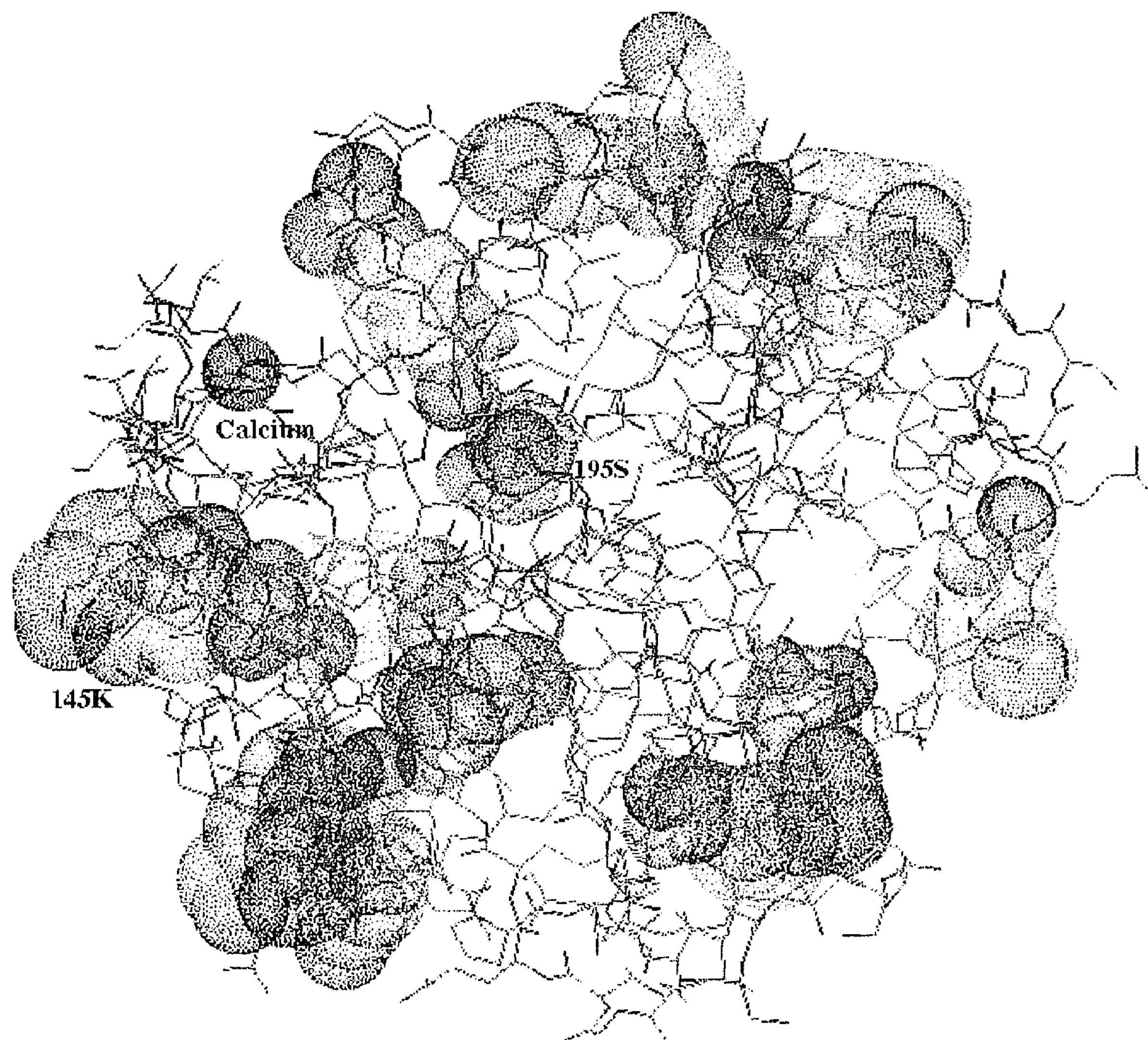


Figure 5.2

Basic Amino Acids of 1tpo

(rotated 90 about x)



Chapter 6

On the Mechanism of Action Of Calcium

6.1 Previous Hypotheses

Since the observation by Bier and Nord in 1951 that calcium slows trypsin autolysis, numerous investigators have advanced hypotheses as to its mechanism of action. Below are listed some of those hypotheses:

1951 Bier & Nord

"Since, however, the presence of calcium does not affect the rate of digestion of other proteins by trypsin, it has to be assumed that the slowing down of the autodigestion takes place via shifting of the existing equilibrium between active and denatured trypsin in the direction of the former."

1953 Nord & Bier

"The addition of calcium ions in sufficient concentration fully prevents the aggregation reducing the sedimentation constant to its normal value, namely that of the monomeric enzyme. It appears, therefore, that calcium, bound to the carboxyl groups of the enzyme, prevents the interaction of enzyme molecules which results in the formation of dimeric or higher polymeric forms."

1971 Stroud, Kay & Dickerson

"The relatively firmly bound calcium ion is common to both trypsinogen and trypsin, inducing a conformational change which stabilizes the proteins to most kinds of denaturation and autolysis."

1975 Bode & Schwager

"Knowing the calcium position, however, it is still difficult to explain the well known stabilizing effect of calcium ion on trypsin . . . it seems reasonable to assume that the calcium makes the loop 68-80 more rigid. It prevents the interaction of charged carboxyl groups with the solute and may stabilize other neighboring regions including the loops containing the alpha-splitting site . . . and another preferred site between Arg-117 and Val-118."

1977 Levilliers, Peron-Renner & Pudles

" . . . calcium ion is known to be a specific ligand that induces upon binding a conformational change which stabilizes the enzyme against autolysis . . . "

1981 Vajda & Garai

"The Mn^{2+}or Ca^{2+} ion bound to trypsin is supposed to control the conformation and thereby the stability and activity of the enzyme.

The Lys145-Ser146 peptide bond, the point of scission in beta to alpha transition, is part of the Ile16 binding pocket, and the Trp141 represents a neighbor of the pocket. Wright showed the important role of the conformation of P3 and P4 residues of the peptide chain of substrate in the binding to the secondary binding site of trypsin. Therefore, the calcium or manganese ions seem likely to interfere with the trypsin autolysis on two routes: (a) by influencing the stereochemical position of P3 and P4 residues (143-142) of the

Ile16 pocket at the 145-146 peptide bond, and (b) by stabilizing the correct conformation of the specificity pocket of the active site by means of the Ile16 pocket. In other words, the bound metal ion may act by enhancing the stability of trypsin as a substrate or by activating trypsin as an enzyme, or both."

1986 Liebman

" . . . the site for calcium binding occurs within the MMRS [macromolecular recognition surface]. Both electrostatic and steric complementarity within the MMRS, which is important for interaction of the serine proteases with macromolecular substrates or inhibitors, can be directly affected by conformational changes or occlusion of this surface. The specific and tight binding of calcium to this region can be viewed as not only affecting the conformation of the region and potentially the conformation of the enzyme but principally interfering with any complexation process that must utilize the full set of surface features and their resultant physicochemical properties."

1989 Bartunik, Summers & Bartsch

"The mechanism is not known by which removal of the calcium may affect the conformation of the remote autolysis loop region of trypsin over a distance of 20 Å. One may envisage that removal of the calcium ion and its replacement by a water molecule may cause the bending radius of loop A to increase by about 0.5 Å. This, in turn may affect the hydrogen bonds between the calcium binding loop (residues 66 to 80) and the segment 153-154 which help to orient the autolysis loop. As a result, long-range structural changes could be mediated through concerted movement of parts of the tertiary structure, in analogy to the observation of such changes induced in proteinase K, an aspartic protease, by removal of a calcium ion from one binding site (Bajorath, 1989)."

The predominant opinion of these scientists is that calcium is inducing a conformational change to the autolysis loop of trypsin which renders it impervious to autolysis. Support for the conformational change hypothesis historically came from ORD measurements, uv differential spectroscopy, and fluorescence studies (see below). These experiments identified either a change to a "more compact" structure or a change in the environment of a tryptophan residue resulting from calcium binding. However, a systematic analysis of all possible mechanisms of action, utilizing the crystal structure analyses of Chapters 1 and 2, the modeling work of Chapter 3, the FTIR experimental work of Chapter 4, and review of the literature, indicates that calcium's mechanism of action does not involve alteration of the 'substrate' autolysis loop conformation, but is electrostatic in nature. Only an electrostatic mechanism is consistent with all of the evidence presented.

If one assumes that the mechanism of action of calcium in slowing trypsin autolysis is the same as in slowing chymotrypsin autolysis, this hypothesis can be extended to

specific electrostatic repulsion between the 'enzyme' and 'substrate' calciums. If one further assumes that the mechanism of action of calcium in the acceleration of the conversion of trypsinogen to β-trypsin is the same as that in slowing the conversion of β-trypsin to α-trypsin, this conclusion can be extended to a repulsion mechanism that has a directing effect which occurs at the diffusional recognition step.

6.2 Examination of the Possible Mechanisms of Action

Below are outlined all of the possible mechanisms of action of calcium in stabilizing trypsin against autolysis. They are subdivided into 'enzyme', 'substrate', and electrostatic effects. Under each is a brief summary of the pertinent data available to either eliminate or support the possibility.

6.2.1 'Enzyme' Effect

6.2.1.1 Alteration of Catalytic Activity

Calcium cannot be exerting its action via disruption of the catalytic triad and enzymatic activity. Numerous reports (Bode & Schwager, 1975a; Gabel & Kasche, 1973; Bier & Nord, 1951a, 1951b; Sipos & Merkel, 1970) have noted that trypsin retains its activity against both small and large substrates in the presence and absence of calcium.

6.2.1.2 Perturbation of Active Site Conformation

Retention of catalytic activity against macromolecules in the presence and absence of calcium implies that the conformations of the active site and recognition surface are conserved.

Conclusion: The catalytic activity and recognition of trypsin towards small and macromolecular substrates is unaffected by calcium binding. This rules out calcium exerting its action on the 'enzyme' via either disruption of the catalytic triad or the 'substrate' binding site.

6.2.2 'Substrate' Effect

6.2.2.1 Alteration of the Reactivity of the 145K Peptide Bond

A simple, albeit farfetched, explanation of calcium's action could be that the 145K-146S cleavage site becomes chemically inert upon calcium binding. Crystal structure evidence against this hypothesis is that the bond lengths and angles of this site are not atypical. The large distance between the calcium and 145K (about 20 Å) also argues against any effect on the chemistry of the reactive peptide bond. Sedimentation studies, discussed below, also argue against this mechanism.

Conclusion: Crystal structure attributes rule out calcium exerting its activity by alteration of the chemistry of the autolysis site.

6.2.2.2 Alteration of the Adaptability of the Autolysis Loop

Another, more complex, explanation is that calcium changes the adaptability of the autolysis site and that after recognition, the 'substrate' is not able to adapt its conformation to the 'enzyme' active site as it migrates into it. This argument is refuted by B value and conformational variability analyses of the autolysis site. The B values of the P1'-P5' residues are among the highest of the trypsin crystal structures and were shown to undergo conformational changes in three inhibited forms. These residues were also shown to be responsible for the preponderance of overlap in the modeled autolysis dimers of Chapter 3. This implies that they must adapt to form the Michaelis complex and is inconsistent with the 'rigidifying' hypothesis.

Furthermore, aggregation experiments performed by Bier et al. (1952), Cunnigham et al. (1953), and Nord & Bier (1953) showed that calcium binding completely prevents the association of trypsins. If the chemistry or adaptability of the autolysis site was changed upon calcium binding, trypsin would, most likely, still aggregate and exhibit a sedimentation velocity greater than that of the monomer.

Conclusion: Crystal structure attributes and sedimentation velocity experiments rule out calcium exerting its activity by alteration of the adaptability of the autolysis site.

6.2.2.3 Alteration of the Autolysis Site Conformation

This is the generally accepted hypothesis today. It assumes that calcium exerts its action by forcing the autolysis loop of the 'substrate' into a conformation which cannot overcome the activation barrier to bind to the active site of the 'enzyme' in the Michaelis complex preceding hydrolysis.

Evidence for the conformational change hypothesis has come from several experimental studies on the effects of calcium binding to trypsin. These studies can be divided into three categories:

1) increased resistance to denaturation.

2) evidence that trypsin takes on a "more compact" structure;

3) identification of change in the environment of a tryptophan residue; and

6.2.2.3.1 Change in Susceptibility to Denaturation

Decreased susceptibility of trypsin to denaturation with calcium binding has been found under the following conditions:

a. increased temperature (Gabel & Kasche, 1973);

b. 8 M urea (Gomez et al., 1977);

c. 4 M guanidine HCl (Levilliers et al., 1977); and

d. chlorambucil (Griffiths & Brecher, 1973).

Conclusion: These results can be construed as evidence that calcium is changing the conformation of trypsin. However, decreased susceptibility to denaturation upon ligand binding is a general phenomena in trypsin (Levilliers, 1977) and other proteins (Jaenicke, 1987) and is not necessarily the result of a conformational change to trypsin, or its autolysis site.

6.2.2.3.2 Change to a "More Compact" Structure

Three papers have historically been cited for evidence that calcium binding produces a "more compact" trypsin structure:

a. Lazdunski and Delaage (1965) concluded from optical rotary measurements that calcium binding "induces a conformational change in the trypsin molecule resulting in a more compact structure."

b. Sipos and Merkel (1970) found that calcium at pH 7.8 and 20 - 40° C "caused the development of positive ultraviolet differential spectrum." They concluded that this "indicated a conformational change to a more compact structure and formation of a calcium-enzyme complex." They also proposed that "there is a progressive opening of the trypsin structure, and in the final step, the unfolded molecules readily aggregate or are enzymatically hydrolyzed unless protected by either H^+ or OH^- concentrations."

c. Gabel and Kasche (1973) found that "Fluorescence measurements as well as the temperature dependence of the autolysis rate of free β-trypsin indicate a more loose structure above 40°." From their fluorescence measurements they "suggest that the trypsin molecule acquires a more compact structure upon binding of calcium."

Conclusion: The assertions that trypsin becomes a "more compact" structure upon calcium binding do not necessarily imply that the autolysis loop conformation is specifically perturbed. The "compactness" probably results from the favorable electrostatic interaction of the positively charged calcium ion with the four negatively charged acidic amino acids which comprise the calcium binding site. This binding most likely 'tightens' the calcium binding loop alone which leads to the experimental results. Moreover, this is a general phenomena in proteins (Jaenicke, 1987).

6.2.2.3.3 Spectral Changes Attributed to a Tryptophan Residue

At least three reports exist in the literature on the effect of calcium binding to the fluorescence spectra of trypsin:

a. Sipos & Merkel (1970), in a much-cited paper, concluded that the differences in the uv spectrum of bovine trypsin at pH 7.8, in the presence of calcium, "are the result of an environmental change of the tyrosine and tryptophan chromophores . . . Calcium reduces interactions between charged residues and causes a shift of the tyrosine and tryptophan chromophores from a polar environment (aqueous) to a nonpolar environment in the interior of the molecule."

b. Matsushima et al. (1971), studying porcine trypsin at pH 8, found maxima at 278, 288, and 299 mu and a shoulder at 294-295 mu in the difference spectra. They concluded that " . . . spectral changes near 300 mu were interpreted as due to an environmental change of tryptophan chromophores" and that it was " . . . likely that one tryptophan takes part in the spectral changes induced by calcium" (from δE_{299} as a function of calcium concentration). These authors also note that bovine trypsin is more labile than porcine trypsin and that the results of Sipos and Merkel (1970) could be due to autolysis effects. Two points of debate in this work are:

1. Porcine trypsin does not autolyze significantly (Vithayathil et al., 1961) and is thus not protected by calcium binding as is bovine trypsin; and

2. Arrio et al. (1973) found no influence of the intrachain splits of the β-, α-, and ψ- forms of trypsin on the environment of tryptophan residues, as measured by fluorescence and phophorescence of bovine trypsin. 8M urea and 0.5% dodecyl sulfate were found to induce a red shift in the emission spectra. The kinetics of this red shift in the presence of 8M urea showed that the first order rate constant for α-trypsin is six times greater than β-trypsin, while the denaturation of ψ-trypsin was found to be too fast to measure.

c. Gabel & Kasche (1973) found at pH 8.1 that "a considerable shift of the maximum of fluorescence emission to longer wavelengths occurred in the presence of calcium at 60°." They also noted that this result "can be caused by autolysis" because the maximum of fluorescence was not reached until after five minutes, which is sufficient time

]for considerable autolysis to have occurred at the concentrations used (2 μM). They concluded that "Owing to the interference of autolysis with the fluorescence measurements nothing can be said about the fluorescence properties in the absence of both calcium and substrate."

Conclusion: Experimental evidence which indicates that the environment of a tryptophan residue changes upon calcium binding is the best argument for the hypothesis that calcium exerts its action by inducing a structural change to the 145K site. The pH of all three experiments rules out a tyrosinate residue (pK of tyrosine = 9.7), instead of a tryptophan, as being responsible for the results. Of the four tryptophan residues in trypsin, 141W is the closest to the calcium (see Appendix B and Figure 6.1) and is the only one with a hydrogen bond to the calcium binding loop. The assignment of this residue from the uv experiments thus seems reasonable. 51W is 20 Å from the calcium, while 215W and 237W are 26 and 32 Å away, respectively. However, results of Chapter 2 showed that the environment of 141W (the P5 residue of the β-trypsin autolysis site) remains constant in the seven crystal structures of the library. This lack of environmental change of the 141W residue in the seven trypsin crystal structures examined, especially relative to the 3ptp, 1tpa, and 2ptc structures - all of which were crystallized in the absence of calcium (see Chapter 2), and to 1tpo, which was crystallized at pH 5 (the pK of the calcium binding site), is indicated by both the hydrogen bonding pattern and vdW contact analyses. 141W is hydrogen bonded to one of the residues of the calcium binding site and alteration of this hydrogen bond upon calcium binding is very likely the cause of the red shift found. A possible explanation of the observed data is that the dipole of the tryptophan excited state interacts with solvent, with this dipole-dipole coupling reducing the energy of the excited state, resulting in emission of light of longer wavelength (lower energy).

6.2.3 Lanthanide Luminescence

Three studies have been performed on terbium luminescence with binding to both porcine and bovine trypsin. Although these studies do not shed any light on the effect of lanthanide [calcium] binding to the trypsin structure (the experiment without lanthanide is uninformative), these studies led to useful information on the nature of the site. The first study, outlined below, was performed before the location of the calcium binding site was unequivocally known. It, therefore, helped to corroborate its identity. The energy transfer between terbium and a tryptophan residue, in all three of the studies outlined below, corroborated the assignment of 141W as the tryptophan residue whose environment changes upon calcium binding:

1. Epstein et al. (1974) found the fluorescence of terbium to be enhanced 10,000 fold when bound to porcine trypsin. The affinity of lanthanide ions was found to depend strongly on their ionic radius. Moreover, they found energy transfer between terbium and a tryptophan residue because maximal terbium fluorescence was obtained when the complex was excited at 295 nm.

2. Epstein et al. (1977) also found the same energy transfer between terbium and a tryptophan residue in porcine, and to a lesser extent, bovine trypsin. Fluorescence enhancement, when the complexes were excited in the range of 280-310 nm, led to this conclusion. Furthermore, they specifically identified the tryptophan residue as 141W (about 10 Å from the Tb^{3+}).

3. DeJersey et al. (1980) found a large enhancement of the green luminescence of terbium ion upon binding to porcine and bovine trypsin, as well as bovine α-chymotrypsin. They also noted the same energy transfer, as above, between terbium and a tryptophan residue and measured binding strengths to various serine proteases.

6.3 Electrostatic Effect

The following observations, either directly or indirectly (by not supporting the conformational change hypothesis), support the hypothesis that calcium inhibits autolysis through a specific electrostatic repulsion that prevents recognition and therefore association:

1. Activity towards a plethora of proteins which contain basic amino acids, but have different secondary and tertiary structures, indicates that trypsin is 'robust' and is capable of cleaving sites with different conformations.

2. FTIR spectroscopy showed no change in the 1644 and 1655 cm^{-1} bands of two inhibited trypsins upon calcium binding (see Chapter 4).

3. α-Trypsin is protected from autolysis by calcium (Abbott et al., 1975). Analyses in Chapters 2 and 5 showed that the β– and α–trypsin autolysis sites have distinctively different topographies and interactions with the calcium binding loop. The α-trypsin autolysis site would also have to undergo a conformation change if the mechanism of action of calcium is the same in slowing both β– and α-trypsin autolysis.

4. McDonald and Kunitz (1941) showed that calcium directs the processing of trypsinogen to β-trypsin. Inert products were formed if calcium was not present. This shows that calcium has a directing effect on the processing. This supports the contention that calcium has a directing effect at the diffusional recognition level in β-trypsin autolysis if the mechanism of action of calcium in accelerating trypsinogen processing is the same as in slowing β-trypsin autolysis. Similarly, Sipos & Merkel (1967) found that calcium binding increases the rate of hydrolysis of 6K-7S and decreases the rate of formation of inert proteins.

5. Chymotrypsin is also protected from autolysis by calcium (Wu & Laskowski, 1956). Since chymotrypsin is a highly homologous serine protease, its mechanism of action of calcium in slowing autolysis is most probably the same. This rules out the possibility that calcium is acting as a 'decoy' by diverting the calcium to the active site (where non-productive binding occurs) since chymotrypsin is specific for uncharged amino

acids. This 'decoy' mechanism could occur in trypsin, which has specificity for positively charged amino acids. Since the only thing in common between trypsin and chymotrypsin is the calciums bound to the 'substrate' and 'enzyme', this implies that it is direct repulsive interaction between the two calciums that is responsible for calcium slowing chymotrypsin autolysis, and by analogy, β-trypsin.

6. Shearwin and Winzor (1990) showed that calcium's effect in preventing chymotrypsin aggregation is not a general electrostatic effect which obeys the Verwey-Overbeek equation. They concluded that its mechanism is shifting of the equilibrium between the monomer and dimer to the monomer. Again, assuming the the mechanism of action of calcium in slowing trypsin and chymotrypsin autolysis is identical, this implies that the mechanism is not of a general electrostatic nature in trypsin.

7. Chiancone et al. (1985) found that the activation domain has no effect on the calcium loop as shown by ^{43}Ca and ^{113}Cd nmr studies. The distance between the calcium and the active site is about 20 Å, about the same as the distance of the calcium to the autolysis site. Similarly, benzamidine or alkylamine binding to the active site was shown not to affect terbium emission intensity (DeJersey et al., 1980).

8. Bier et al. (1952) and Nord & Bier (1953) found from ultracentrifugation experiments that calcium prevents the aggregation of trypsin. Cunningham et al. (1953) showed that trypsin dimerizes at pH 5 but does not dimerize at pH 6.28 and 7.81 with $CaCl_2$. At pH 5 it was shown to dimerize to a lesser extent than without calcium. Epstein et al. (1974) later showed that the pK of calcium binding is around 5. This helps explain the finding of increased sedimentation with decreasing pH.

9. The DIP-inhibited (1tpo) and PTI-inhibited (2ptc, 1tpa) structures of trypsin are probably not bound by calcium (see Chapter 2) and exhibit only minor conformational differences from the calcium bound structures - which are probably due to active site inhibition. These differences are not of the order needed for productive binding (see Chapter 3).

10. B values and solvent accessibilities of the autolysis site (with calcium bound) are among the highest in the trypsin crystal structures. If calcium affects the autolysis site conformation by making it inaccessible or 'burying' it, the B values and solvent accessibilities of this area would be expected to be low.

11. The sparse hydrogen bonding pattern between the calcium binding loop and the remainder of the protein (see Chapter 2) argues against calcium binding propagating an effect to the autolysis loop or remainder of the protein.

12. Liebman (1986) found calcium to be part of the macromolecular recognition surface in trypsin and hypothesized that complexation might "utilize the full set of surface features and their resultant physicochemical properties." This implies that calcium's electrostatic features have had an effect of trypsin's evolution and its physiological control.

13. Modeling of the autolysis dimer in Chapter 3 showed that if the active-site remains conserved, the 'substrate' changes needed for formation of the Michaelis complex are greater than any of those seen in the seven trypsin crystal structures.

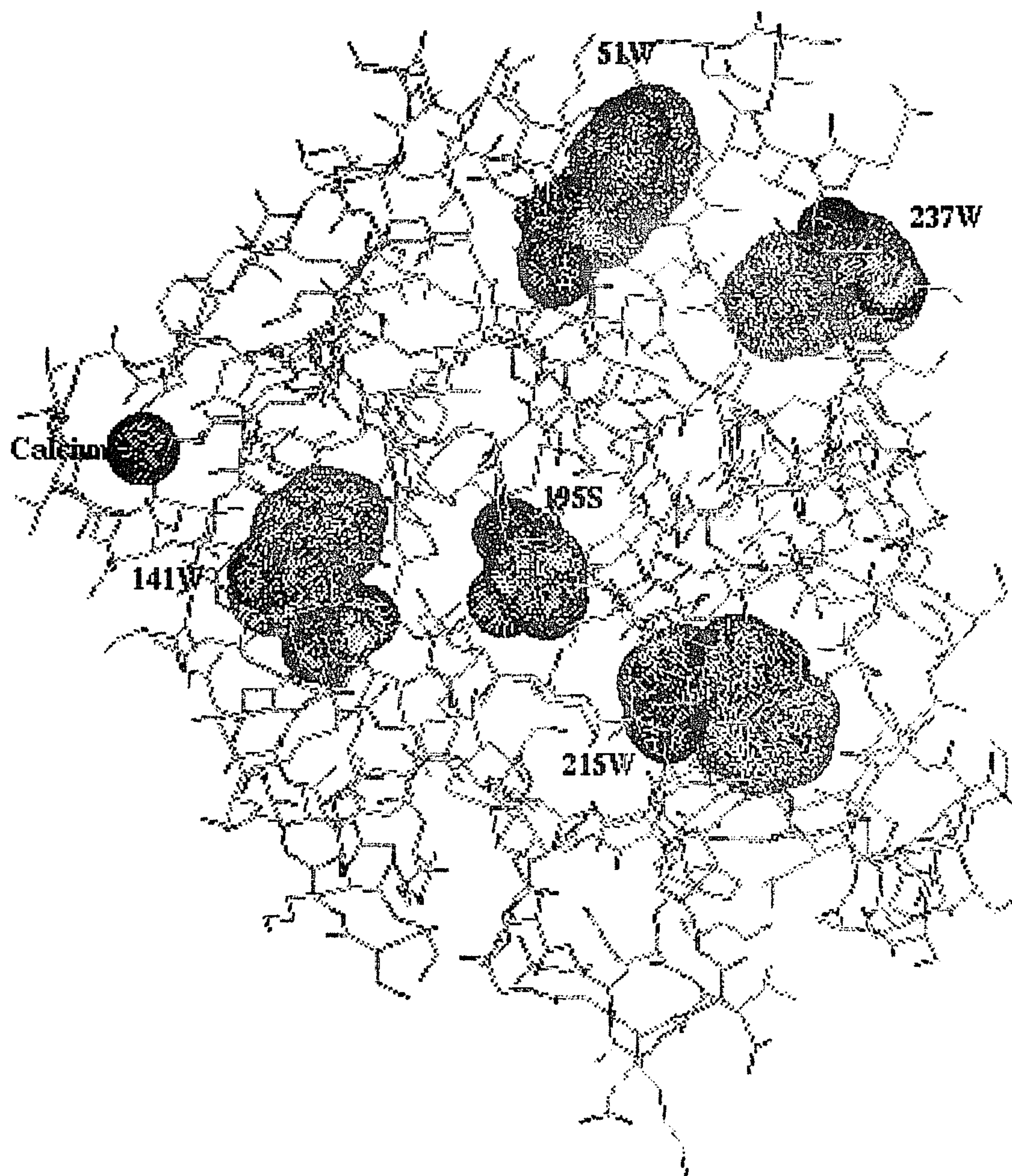
14. Molecular dynamics simulations performed by Brunger (1987) to 200 psec, with solvent, show no large changes in the conformation of the autolysis loop.

Conclusion: A process of elimination, made possible by crystal structure analyses, modeling, and experimental work, indicate that it is likely that the mechanism of action of calcium in slowing trypsin autolysis is electrostatic repulsion between 'enzyme' and 'substrate' calciums at the diffusional recognition step. Evidence was shown not to support the hypotheses that calcium exerts its activity by either affecting the 'enzyme', altering the reactivity of the scissile peptide bond, or changing the adaptability of the autolysis site. The alternative, that calcium acts by inducing a conformational change in the autolysis site, is supported mainly by fluorescence measurements which have identified a change in the environment of a tryptophan residue (most probably 141W). However, FTIR spectroscopic analyses of calcium's effect on two inhibited β-trypsins (see Chapter 4) refute this. This, along with the recently published sedimentation equilibrium study on

calcium binding to chymotrypsin, the observation that α-trypsin is also protected from autolysis by calcium, the early findings that calcium prevents trypsin aggregation and directs processing of trypsinogen to active products, analyses of the attributes of trypsin's three physiological sites in Chapter 2, and the computational modelling work presented in Chapter 3, support the hypothesis that calcium slows trypsin autolysis via a specific electrostatic mechanism which prohibits the 'enzyme' and 'substrate' from associating.

Figure 6.1

Tryptophan Positions of 1tpo



Conclusion

β-trypsin is a typical globular protein with backbone conformational and thermal variability attributes similar to other proteins whose crystal structures have been solved to date. Its active site is particularly well-conserved, while its calcium binding and autolysis loops exhibit progressively greater conformational and thermal variability. Specificity of β-trypsin autolysis for the 145K site results from both its high solvent accessibility and thermal variability, and its minimal steric overlap as determined by a computational screening procedure. Analyses of the 16 basic amino acids of β-trypsin as potential substrates in its autolysis suggest that cleavage of β-trypsin induces a conformational change in the 188K site of α-trypsin which is necessary for subsequent processing. It also suggests that mobility of the P1'-P5' residues is necessary for allowing bulk water to enter the active site for the deacylation step of autolysis.

Computational modeling of the autolysis dimer showed that the P1'-P5' residues of the 'substrate' autolysis site are responsible for the majority of the steric overlap between the two proteins and that the backbone of these residues must adapt themselves considerably to allow for formation of the Michaelis complex. The extent of this adaptation, as shown by molecular mechanics minimizations, is larger than the crystal changes induced by diisopropylfluorophosphate or PTI inhibition. If calcium slows autolysis by the generally accepted 'conformational change' hypothesis, it would most likely be observed by FTIR spectroscopy. This method indicated, however, that calcium binding does not change the [autolysis loop] backbone conformation of two inhibited forms of bovine β-trypsin.

Crystal structure analyses, molecular modeling, and FTIR evidence presented here, along with the observations of several others on trypsin and chymotrypsin biochemistry, biophysics, and autolysis, suggest that calcium's mechanism of action is of a specific electrostatic origin. Moreover, the level of action probably occurs via an electrostatic repulsion between the 'enzyme' and 'substrate' calciums at the diffusional recognition step.

Appendix A

Unit Cell Contacts

Residue	1tpo Total	1tpo Backbone	1tpo Back-Back	3ptn Total	3ptn Backbone	3ptn Back-Back
20Y	51	0	0			
24A	3	3	3			
25N	6	6	2			
26T	40	16	3			
28P	1	0	0			
34N	8	0	0	8	0	0
37S	1	1	0	99	6	0
38G	40	40	5	40	40	7
39Y	106	10	0	99	6	0
40H	1	1	0	1	1	0
49S				14	7	7
50Q				49	5	5
51W				1	0	0
59Y	82	0	0	83	0	0
60K	3	1	0	7	2	0
61S	4	2	0	2	1	0
64Q	1	0	0	1	0	0
66R	23	0	0	22	0	0
72N	12	0	0	13	0	0
74N	98	30	3	95	29	1
75V	10	2	0	8	2	0
76V	2	0	0	4	0	0
82F	2	0	0	3	0	0
86S	63	42	22	49	37	18
87K	39	11	5	41	11	5
88S	19	15	0	21	14	0
89I	23	9	0	21	0	0
90V	34	14	4	31	12	3
91H	1	1	1			
92P	52	24	6	53	22	6
93S	2	2	0	2	2	0
94Y	2	2	0	4	3	0
96S	7	7	0	8	5	1
97N	72	29	0	25	2	0
98T	8	6	0	1	0	0
99L	5	1	0	3	0	0
107K				2	0	0
109K	11	0	0	7	0	0
111A				2	0	0
114L				49	24	18
115N				7	4	0
116S	21	8	1	4	0	0
117R	13	2	0	4	0	0

Appendix A (cont'd)

Residue	1tpo Total	1tpo Backbone	1tpo Back-Back	3ptn Total	3ptn Backbone	3ptn Back-Back
122S	1	0	0			
125T	24	3	3	57	32	1
127S	9	2	0	44	17	5
128C				2	1	1
130S				11	2	1
132A				3	3	0
133G				4	4	0
134T				16	6	0
135Q				5	1	0
137L				1	0	0
146S	4	4	0			
147S	21	13	0			
148G	18	18	6			
149T	36	21	8	29	16	7
150S	24	17	11	13	10	8
151Y	55	17	13	55	15	13
153D	30	3	0	34	4	0
159K	42	2	0	10	0	0
165D				30	5	5
166S				39	19	14
169K				51	8	2
170S	6	6	0	5	4	2
171A	3	3	0	2	2	0
172Y				14	2	0
173P	40	13	5	17	7	0
174G	7	7	1	53	53	14
175Q	28	2	0	36	9	0
176I				11	11	0
177T				7	4	0
192Q	12	0	0			
201C				1	1	1
202S	20	16	6	32	23	13
203G	16	16	14	15	15	5
204K	16	0	0	5	0	0
215W				8	0	0
216G				5	5	3
217S	10	8	8	22	9	7
219G	17	17	16	1	1	0
220C	1	1	1			
221Q	6	0	0			
224K	2	0	0	15	0	0
237W	18	0	0	16	0	0
240Q	21	9	0	28	7	0
241T	33	16	0	28	11	0
242I				8	6	0
243A				63	51	23
244S	26	15	0	61	39	3
245N	23	0	0	36	0	0

Appendix B

TRYPSIN SUPERPOSITION RESULTS

cont = contacts to PTI of 2ptc (* = backbone cont, underlined = P5-P1 cont)
Ca, 145K, 195S are distances to Ca^{++}, 145K, and 195S in 1tpo
bold values are residues determined as outliers by Chi-square test

1tpo -	3ptn	223 = .26	204 = .18	1tpo -	2ptc	223 = .34	215 = .30
	3ptb	223 = .11			1tpa	223 = .34	214 = .30
	1tpp	223 = .11					
	3ptp	223 = .21	211 = .14				

aa	cont	Ca	145K	195S	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
16I		18	8	10	.09	.06	.05	.15	.17	.18
17V		21	6	13	.09	.13	.10	.12	.07	.06
18G		23	8	16	.19	.21	.16	.22	.22	.24
19G		20	11	16	.16	.04	.19	.14	.34	.34
20Y		18	13	18	.14	.08	.05	.17	.14	.20
21T		15	14	18	.22	.11	.10	.16	.27	.24
22C		14	17	17	.16	.11	.07	.13	.26	.23
23G		13	20	20	.19	.05	.11	.11	.32	.27
24A		10	22	20	.24	.13	.13	.16	.31	.25
25N		10	23	19	.19	.14	.08	.09	.28	.27
26T		14	23	19	.13	.09	.10	.14	.27	.28
27V		14	22	16	.06	.03	.05	.19	.12	.10
28P		13	25	17	.08	.07	.13	.07	.11	.07
29Y		15	25	15	.16	.06	.08	.13	.11	.03
30Q		12	21	12	.15	.13	.05	.16	.12	.08
31V		11	22	12	.15	.12	.06	.12	.10	.11
32S		10	20	10	.18	.09	.16	.12	.15	.22
33L		13	21	10	.13	.07	.12	.06	.22	.26
34N		12	22	13	.09	.07	.10	.15	.22	.20
37S		15	24	15	.14	.03	.04	.15	.47	.51
38G		14	24	17	.17	.11	.09	.13	.45	.42
39Y	74*	12	20	14	.15	.10	.17	.18	.35	.37
40H	27*	11	18	11	.12	.05	.09	.15	.32	.27
41F	60*	15	17	8	.07	.05	.04	.06	.16	.09
42C	14*	15	17	5	.16	.08	.08	.06	.15	.14
43G		14	18	6	.11	.09	.08	.07	.21	.18
44G		16	21	8	.13	.16	.19	.10	.18	.12
45S		17	24	11	.11	.05	.06	.09	.19	.14
46L		17	27	15	.15	.06	.11	.08	.11	.07
47I		21	30	17	.08	.04	.06	.11	.13	.11
48N		21	33	20	.15	.07	.10	.06	.11	.13
49S		20	34	21	.13	.07	.14	**.39**	.17	.17
50Q		20	34	21	.36	.10	.03	.14	.21	.11
51W		20	31	17	.11	.04	.09	.10	.16	.11
52V		19	27	14	.10	.03	.06	.05	.12	.17
53V		20	26	11	.08	.02	.08	.10	.10	.10
54S		20	23	8	.15	.05	.02	.12	.17	.14
55A		22	22	7	.22	.05	.05	.10	.15	.22

Appendix B (cont'd)

aa	cont	Ca	145K	195S	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
56A		25	24	10	.05	.06	.11	.10	.10	.08
57H	85*	25	22	8	.21	.03	.06	.21	.33	.20
58C	5*	21	21	7	.23	.10	.10	.14	.31	.26
59Y		23	25	11	.12	.06	.12	.16	.25	.26
60K	11	22	26	13	.12	.06	.12	.25	.29	.22
61S		22	29	17	.28	.17	.19	.26	.37	.45
62G		20	29	18	.30	.14	.16	.07	.38	.55
63I		17	27	15	.16	.19	.15	.16	.11	.20
64Q		14	27	16	.11	.13	.14	.17	.32	.26
65V		12	26	15	.11	.08	.12	.25	.33	.35
66R		9	24	15	.05	.06	.01	.08	.11	.18
67L		9	25	16	.10	.08	.12	.26	.10	.11
69G		7	25	18	.38	.21	.12	.31	.13	.05
70E		5	22	17	.11	.13	.07	.13	.08	.11
71D		5	20	17	.09	.06	.05	.08	.13	.08
72N		4	19	18	.15	.09	.09	.10	.20	.15
73I		4	19	16	.17	.08	.10	.16	.22	.21
74N		6	20	19	.19	.15	.19	.20	.36	.33
75V		4	23	22	.40	.24	.21	.12	.56	.61
76V		4	26	23	**.46**	.14	.11	.17	.45	.56
77E		5	26	24	**.44**	.12	.09	.26	.50	.50
78G		7	29	25	**.61**	.19	.08	**.40**	**1.14**	**1.17**
79N		8	28	23	.38	.10	.17	.11	.67	**.71**
80E		6	27	21	.12	.12	.07	.24	.28	.32
81Q		9	29	21	.16	.03	.03	.13	.26	.25
82F		10	29	20	.20	.05	.09	.15	.28	.21
83I		14	31	20	.13	.07	.06	.11	.28	.26
84S		16	31	19	.03	.07	.02	.11	.30	.24
85A		19	31	18	.11	.05	.07	.09	.19	.16
86S		22	34	21	.05	.16	.07	.07	.20	.27
87K		24	34	20	.14	.04	.06	.11	.31	.32
88S		24	31	16	.17	.06	.06	.14	.36	.36
89I		27	32	17	.09	.07	.09	.05	.25	.19
90V		28	30	16	.08	.12	.15	.13	.32	.41
91H		30	31	15	.06	.12	.11	.08	.16	.11
92P		33	34	19	.26	.12	.07	.11	.31	.35
93S		35	33	19	.19	.12	.12	.07	.31	.29
94Y	2	32	29	15	.09	.07	.06	.16	.12	.03
95N		33	28	16	.15	.05	.06	.13	.42	.41
96S	10*	33	25	14	.21	.09	.09	.21	.61	.61
97N	19*	31	25	16	.30	.05	.04	.15	.60	**.69**
98T	11*	34	25	15	.14	.04	.04	.05	.55	**.73**
99L	36*	31	23	12	.14	.04	.07	.05	.42	.53
100N		33	26	14	.10	.09	.03	.07	.40	.34
101N		31	27	14	.07	.03	.09	.12	.22	.24
102D	1	27	24	10	.18	.05	.03	.04	.22	.17
103I		26	26	11	.20	.09	.08	.14	.27	.24
104M		24	27	12	.17	.15	.11	.07	.23	.18
105L		23	29	14	.09	.06	.10	.17	.27	.27

Appendix B (cont'd)

aa	cont	Ca	145K	195S	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
106I		21	29	15	.14	.05	.05	.02	.23	.14
107K		21	32	18	.07	.07	.09	.12	.13	.10
108L		19	32	19	.06	.04	.05	.14	.31	.18
109K		20	35	23	.17	.07	.06	.06	.42	.44
110S		20	36	24	.18	.12	.06	.20	.42	.39
111A		19	36	24	.35	.06	.14	.13	.33	.30
112A		16	33	22	.15	.12	.03	.12	.36	.41
113S		16	35	25	.08	.06	.04	.14	.31	.26
114L		17	33	24	.13	.04	.05	.16	.39	.30
115N		17	33	25	**.71**	.14	.29	.11	**.88**	**.60**
116S		16	31	25	**1.46**	.07	.13	**.35**	**1.17**	**1.06**
117R		13	28	22	**.98**	.05	.11	.21	**.97**	.88
118V		13	29	20	.33	.12	.08	.13	.34	.29
119A		16	30	20	.24	.11	.11	.27	.13	.18
120S		19	30	19	.15	.10	.06	.11	.28	.19
121I		20	28	17	.14	.09	.08	.12	.19	.17
122S		24	31	19	.12	.09	.16	.10	.16	.21
123L		26	31	18	.18	.05	.05	.07	.23	.23
124P		29	31	20	.29	.07	.08	.11	.25	.29
125T		33	35	23	.12	.12	.09	.07	.29	.29
127S		34	33	23	.42	.09	.08	.22	.22	.23
128C		33	31	21	.12	.06	.04	.13	.21	.24
129A		32	28	20	.32	.05	.07	.14	.45	.36
130S		35	29	22	**.42**	.04	.12	.16	.39	.40
132A		35	26	21	.31	.15	.11	.17	.44	.46
133G		34	26	22	.30	.16	.07	.11	.42	.66
134T		31	25	21	.07	.05	.07	.11	.24	.10
135Q		28	23	19	.30	.13	.06	.15	.09	.11
136C		25	21	16	.11	.10	.09	.05	.09	.17
137L		21	19	15	.16	.06	.03	.07	.15	.17
138I		19	16	11	.10	.10	.05	.15	.11	.14
139S		16	15	10	.15	.06	.03	.10	.22	.19
140G		13	13	8	.12	.10	.11	.16	.19	.11
141W		12	12	9	.04	.08	.12	.11	.22	.15
142G		15	9	8	.15	.11	.09	.10	.26	.15
143N		18	6	11	.12	.12	.14	.13	.24	.20
144T		19	4	13	.10	.12	.03	**.42**	.25	.22
145K		22	0	15	.28	.12	.08	**.43**	.39	.29
146S		26	4	16	.23	.08	.19	**1.57**	.60	.54
147S		27	5	18	.27	.04	.04	**1.16**	.49	.59
148G		25	6	18	.29	.14	.05	**.52**	**.96**	**1.08**
149T		21	6	16	.18	.13	.21	**.49**	**.74**	.64
150S		18	6	16	.15	.14	.14	.25	**.70**	.52
151Y	44	15	8	14	.10	.13	.14	.06	.40	.42
152P		13	10	15	.23	.13	.12	.17	.35	.33
153D		9	14	16	.24	.20	.15	.21	**.90**	**.77**
154V		9	15	16	.10	.11	.11	.21	.52	.46
155L		10	15	13	.03	.08	.11	.16	.17	.14
156K		14	12	13	.17	.04	.05	.08	.20	.21

Appendix B (cont'd)

aa	cont	Ca	145K	195S	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
157C		17	14	14	.11	.05	.09	.11	.19	.13
158L		21	14	14	.13	.10	.11	.08	.29	.25
159K		23	17	16	.25	.08	.07	.07	.27	.20
160A		26	17	16	.11	.01	.07	.05	.32	.29
161P		29	20	18	.06	.03	.09	.17	.24	.09
162I		31	22	17	.02	.06	.05	.09	.13	.11
163L		34	22	19	.15	.09	.10	.17	.50	.43
164S		37	26	21	.36	.08	.08	.07	.41	.35
165D		38	26	21	**.57**	.03	.09	**.37**	.35	.35
166S		40	27	24	**.57**	.11	.20	.14	.51	.60
167S		38	24	22	**.46**	.09	.14	.10	.54	.61
168C		36	23	19	.33	.05	.08	.11	.37	.27
169K		39	25	21	.34	.12	.17	.20	.26	.13
170S		40	25	23	.51	.16	.23	.29	.35	.32
171A		37	21	20	**.65**	.11	.09	.09	.33	.33
172Y		37	22	19	**.53**	.06	.14	.15	.26	.27
173P		40	25	22	**.50**	.06	.13	.25	.29	.39
174G		41	27	22	**.53**	.09	.13	.15	.22	.48
175Q	5	37	25	18	.34	.08	.13	.08	.18	.23
176I		36	25	17	.31	.16	.11	.24	.28	.22
177T		37	28	18	.25	.08	.07	.08	.28	.21
178S		37	29	19	.28	.20	.12	.06	.17	.19
179N		33	28	16	.16	.11	.04	.06	.05	.03
180M		32	24	14	.12	.05	.08	.14	.21	.22
181F		31	23	14	.04	.06	.15	.12	.14	.05
182C		31	20	14	.12	.10	.09	.14	.12	.13
183A		29	18	14	.14	.11	.08	.21	.17	.20
184G		28	16	15	.15	.10	.12	.20	.15	.14
184Y		31	16	18	.24	.07	.06	.17	.26	.21
185L		34	17	20	**.46**	.14	.09	.21	.43	.39
186E		34	16	21	.26	.12	.06	.20	.36	.37
187G		31	12	18	.21	.14	.16	.26	.46	.41
188G		28	9	18	.06	.03	.24	.24	.58	.54
188K		25	10	15	.27	.22	.15	.12	.31	.27
189D	8*	24	10	12	.13	.08	.07	.16	.20	.19
190S	22*	21	10	8	.16	.16	.12	.10	.20	.15
191C	28*	21	8	7	.12	.19	.12	.09	.17	.21
192Q	113*	20	10	7	.07	.36	.18	.24	.20	.20
193G	31*	17	12	5	.14	.10	.07	.05	.32	.36
194D	18*	17	12	4	.11	.07	.08	.13	.30	.27
195S	44*	19	15	0	.10	.12	.09	.13	.39	.27
196G		19	19	4	.09	.07	.04	.10	.36	.26
197G		17	18	5	.05	.16	.20	.06	.32	.28
198P		19	20	9	.07	.02	.06	.08	.19	.19
199V		22	20	11	.06	.06	.07	.13	.19	.23
200V		22	23	14	.14	.05	.07	.13	.07	.15
201C		26	25	17	.28	.07	.06	.11	.11	.20
202S		27	28	21	**.67**	.08	.03	.12	.24	.34
203G		24	28	20	**.83**	.09	.06	.18	.24	.18

Appendix B (cont'd)

aa	cont	Ca	145K	195S	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
204K		25	28	18	.39	.11	.13	.15	.34	.34
209L		23	26	15	.16	.06	.04	.12	.29	.32
210Q		26	25	14	.14	.03	.09	.07	.20	.22
211G		25	22	11	.10	.15	.18	.17	.14	.15
212I		22	20	7	.09	.06	.12	.12	.09	.05
213V	6*	22	17	5	.14	.08	.12	.11	.15	.11
214S	31*	24	18	6	.11	.09	.13	.05	.30	.19
215W	75*	26	16	7	.07	.18	.06	.11	.24	.18
216G	31*	28	14	10	.13	.15	.25	.21	.17	.21
217S		30	14	13	.30	.22	.14	**.38**	.40	.30
219G	6*	28	10	13	.22	.22	.09	**.43**	.24	.19
220C	8*	26	8	12	.16	.08	.12	.22	.18	.21
221A		27	8	14	.23	.02	.13	.15	.17	.17
221Q		31	11	17	.18	.11	.03	.21	.18	.32
222K		34	14	20	.18	.17	.10	.15	.28	.22
223N		35	17	20	.18	.18	.17	.21	.40	.35
224K		32	15	16	.12	.14	.06	.17	.44	.38
225P		30	15	14	.10	.08	.17	.12	.24	.23
226G	4*	27	14	10	.10	.15	.19	.11	.28	.35
227V	2*	28	18	10	.12	.07	.10	.05	.15	.19
228Y	1	27	19	9	.04	.10	.05	.07	.14	.12
229T		27	23	10	.10	.06	.06	.07	.07	.06
230K		29	26	14	.11	.10	.06	.09	.16	.15
231V		28	27	14	.12	.12	.06	.09	.21	.26
232C		31	30	18	.21	.08	.04	.07	.32	.37
233N		33	31	19	.18	.04	.05	.06	.29	.41
234Y		31	32	18	.18	.03	.07	.12	.37	.32
235V		31	33	20	.07	.07	.08	.10	.32	.30
236S		34	37	23	.07	.03	.04	.17	.48	.43
237W		32	35	21	.20	.07	.06	.17	.36	.26
238I		29	34	19	.12	.06	.04	.05	.17	.19
239K		30	37	22	.19	.08	.03	.04	.34	.40
240Q		33	39	24	.22	.04	.01	.12	.45	.48
241T		30	37	22	.35	.11	.14	.16	.44	.44
242I		28	37	22	.36	.13	.06	.07	.55	.60
243A		31	41	26	.31	.12	.15	.10	.66	**.69**
244S		33	42	27	.24	.07	.03	.25	.29	.36
245N		30	40	25	.19	.16	.13	.17	.28	.34

Appendix C

RESIDUE SOLVENT ACCESSIBILITIES OF ALL TRYPSINS

cont = contacts to PTI of 2ptc (*=backbone contacts, underlined=P5-P1 contacts)

aa	cont	1tpo	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
16I		0	0	0	0	1	1	1
17V		8	8	9	8	2	6	7
18G		41	48	45	45	23	46	48
19G		28	28	30	28	35	33	35
20Y		40	37	40	42	40	39	39
21T		44	52	47	44	50	39	38
22C		12	9	10	12	12	13	15
23G		32	35	34	34	37	34	34
24A		51	55	50	47	37	45	48
25N		21	23	25	19	15	19	18
26T		54	58	54	53	48	53	54
27V		7	4	5	5	5	7	7
28P		23	21	25	23	26	21	21
29Y		9	11	8	8	9	9	9
30Q		6	5	6	6	6	3	3
31V		1	0	1	1	1	1	0
32S		0	0	0	2	2	2	1
33L		0	0	0	0	0	0	0
34N		10	9	7	13	7	9	11
37S		25	22	22	25	25	26	26
38G		80	81	75	76	66	78	77
39Y	74*	52	51	51	52	50	54	54
40H	27*	17	20	17	19	19	16	17
41F	60*	15	17	16	14	15	17	16
42C	14*	6	6	6	4	5	4	6
43G		0	0	0	0	2	0	0
44G		0	0	0	0	0	0	0
45S		0	0	0	0	2	0	0
46L		1	0	1	1	1	1	1
47I		10	8	11	10	9	12	11
48N		30	33	30	30	33	33	33
49S		31	25	29	29	25	16	29
50Q		29	39	30	30	30	34	33
51W		3	3	3	3	4	0	4
52V		0	0	0	0	0	0	0
53V		0	0	0	0	1	0	0
54S		0	0	0	0	0	0	0
55A		0	0	0	0	0	0	0
56A		3	3	1	3	2	3	3
57H	85*	35	35	36	29	31	41	41
58C	5*	5	3	3	4	5	3	3
59Y		52	52	49	53	47	49	50
60K	11	36	32	32	35	31	33	33
61S		74	73	75	76	73	72	72

Appendix C (cont'd)

aa	cont	1tpo	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
62G		72	70	72	68	83	76	73
63I		6	6	6	6	5	5	5
64Q		26	25	20	25	26	25	26
65V		0	0	0	0	0	0	0
66R		22	21	20	22	25	20	19
67L		2	0	2	2	1	0	1
69G		11	9	13	13	10	6	7
70E		10	8	9	10	9	10	9
71D		11	14	11	11	12	15	12
72N		17	16	16	16	12	16	16
73I		17	18	17	21	21	20	19
74N		67	67	65	68	67	63	61
75V		49	47	51	48	51	49	50
76V		71	73	73	71	61	72	68
77E		50	49	50	51	50	49	51
78G		67	67	63	69	55	74	68
79N		30	35	30	34	38	36	38
80E		20	18	19	21	17	21	18
81Q		24	24	19	24	16	22	21
82F		35	38	34	35	29	42	37
83I		13	12	13	13	14	12	13
84S		34	30	32	34	41	31	30
85A		18	22	21	19	23	18	18
86S		51	45	52	48	55	47	43
87K		32	35	30	37	42	38	39
88S		29	32	31	31	27	26	31
89I		22	21	21	22	22	23	23
90V		31	31	26	32	31	27	27
91H		12	13	12	12	12	13	12
92P		69	68	69	67	67	68	69
93S		53	54	52	49	51	51	48
94Y	2	23	23	17	22	22	21	23
95N		40	41	39	40	39	39	39
96S	10*	56	58	58	58	55	58	58
97N	19*	82	82	82	81	84	76	76
98T	11*	49	49	49	49	50	49	49
99L	36*	19	20	20	19	19	25	25
100N		11	19	22	19	21	18	16
101N		11	10	9	12	10	11	11
102D	1	0	1	0	0	1	1	1
103I		0	0	0	0	0	0	0
104M		0	0	0	0	0	0	0
105L		0	0	1	0	0	0	0
106I		0	0	0	0	0	0	0
107K		20	21	19	21	20	21	20
108L		1	3	1	1	1	1	1
109K		62	64	63	62	72	63	64
110S		52	55	52	53	50	49	50

Appendix C (cont'd)

aa	cont	1tpo	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
111A		48	53	49	45	42	44	46
112A		7	6	6	7	2	6	6
113S		64	55	58	64	42	55	54
114L		37	47	40	38	41	44	41
115N		50	42	41	52	48	46	48
116S		73	64	83	72	76	70	72
117R		47	39	33	48	63	43	44
118V		2	1	1	2	2	0	1
119A		22	28	22	24	26	28	27
120S		38	38	38	38	38	35	35
121I		10	9	11	10	10	10	12
122S		43	51	51	46	48	50	48
123L		22	20	23	16	22	18	18
124P		4	5	4	4	4	4	4
125T		83	86	93	82	90	69	76
127S		56	62	58	56	63	60	60
128C		23	42	42	42	43	44	44
129A		24	27	24	22	23	26	26
130S		62	65	61	62	63	58	58
132A		47	48	48	45	37	49	53
133G		67	64	65	65	52	63	58
134T		25	24	27	27	25	22	23
135Q		62	64	65	63	51	59	57
136C		4	2	3	3	4	4	3
137L		21	22	23	21	23	20	20
138I		1	1	1	1	2	0	0
139S		1	1	1	1	1	0	0
140G		0	0	0	0	3	0	0
141W		2	2	2	2	2	2	2
142G		0	0	0	0	0	1	1
143N		2	7	4	1	7	8	8
144T		12	11	14	14	8	19	17
145K		51	48	50	50	38	49	46
146S		41	39	39	32	46	42	41
147S		72	71	72	67	93	81	82
148G		66	62	64	33	62	58	56
149T		71	69	70	54	67	63	64
150S		42	37	39	41	36	35	33
151Y	44	48	50	49	47	48	52	52
152P		14	13	12	16	9	14	15
153D		39	39	41	40	41	44	42
154V		31	23	25	27	27	24	25
155L		5	4	5	41	5	4	5
156K		20	16	19	19	22	19	19
157C		2	1	2	2	2	2	1
158L		1	2	4	3	3	3	3
159K		46	44	49	51	59	51	52
160A		0	2	0	1	1	0	0
161P		27	27	24	25	59	20	19

Appendix C (cont'd)

aa	cont	1tpo	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
162I		6	8	7	6	6	7	6
163L		17	13	16	17	7	14	14
164S		36	38	36	38	28	35	36
165D		57	52	53	55	64	50	51
166S		62	59	65	65	65	61	60
167S		19	18	18	19	28	31	33
168C		1	2	1	1	1	2	1
169K		39	49	39	41	51	47	47
170S		63	63	66	64	62	67	69
171A		12	13	11	13	8	21	22
172Y		1	2	1	1	2	5	5
173P		60	49	61	61	62	63	64
174G		99	63	100	100	100	93	96
175Q	5	48	13	48	47	47	49	48
176I		10	2	11	7	11	12	12
177T		29	66	30	29	29	28	29
178S		59	99	59	64	62	65	68
179N		15	51	14	14	16	16	14
180M		3	14	4	4	7	3	3
181F		9	31	10	10	8	10	9
182C		1	62	1	0	0	0	0
183A		2	16	1	1	1	0	0
184G		6	5	7	6	6	2	4
184Y		32	8	32	28	27	28	26
185L		34	0	35	30	37	37	38
186E		84	1	79	66	83	75	71
187G		14	6	7	18	20	15	13
188G		52	32	50	51	46	42	43
188K		42	28	48	47	45	43	45
189D	8*	4	38	4	4	6	5	4
190S	22*	5	6	0	0	8	9	10
191C	28*	8	8	0	0	3	8	10
192Q	113*	54	57	45	36	69	57	57
193G	31*	20	20	19	12	15	23	24
194D	18*	0	1	0	0	1	0	0
195S	44*	16	17	7	0	0	17	14
196G		0	0	0	0	1	0	0
197G		1	1	1	0	1	0	0
198P		1	1	0	1	1	0	0
199V		0	0	0	0	0	0	0
200V		9	12	10	10	9	8	10
201C		4	2	3	4	2	4	4
202S		79	68	72	78	78	80	83
203G		53	52	47	51	48	47	50
204K		34	39	44	35	21	36	36
209L		0	0	0	0	0	0	0
210Q		6	7	7	7	6	9	7
211G		0	0	0	0	0	0	0

Appendix C (cont'd)

aa	cont	1tpo	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
212I		0	0	0	0	2	0	0
213V	6*	4	3	0	0	1	5	5
214S	31*	3	5	2	1	4	6	7
215W	75*	18	18	13	13	17	21	21
216G	31*	31	30	18	20	36	23	24
217S		41	40	42	42	33	36	39
219G	6*	59	57	47	52	70	39	40
220C	8*	6	6	4	1	20	6	6
221A		8	8	8	9	12	9	9
221Q		40	39	41	38	33	39	39
222K		72	70	69	70	58	65	66
223N		44	47	47	47	54	49	58
224K		31	32	31	31	30	38	40
225P		4	5	4	4	4	4	4
226G	4*	1	1	0	0	3	6	6
227V	2*	0	0	0	0	1	1	1
228Y	1	1	1	1	1	1	1	1
229T		1	1	1	1	3	0	0
230K		30	29	29	30	23	32	31
231V		0	0	0	0	0	0	0
232C		15	14	14	15	11	15	13
233N		43	41	42	42	36	40	41
234Y		42	3	4	4	4	4	55
235V		17	17	16	19	16	18	17
236S		65	73	65	73	53	70	72
237W		18	17	17	18	12	20	21
238I		1	2	1	2	1	1	1
239K		49	60	51	59	61	59	56
240Q		62	61	59	62	63	66	65
241T		10	10	9	10	6	12	11
242I		23	18	22	22	19	18	17
243A		68	69	70	68	71	60	61
244S		56	55	55	58	67	59	55
245N		31	31	32	29	28	27	29

Appendix D

ALPHA CARBON B VALUES OF ALL TRYPSINS

cont = contacts to PTI of 2ptc (*=backbone contacts, underlined=P5-P1 contacts)
sas are solvent accessibilities of 1tpo

	mean	range
1tpo	16.2 ± 4.0	9 - 31
3ptn	14.5 ± 6.3	5 - 43
3ptb	13.0 ± 4.6	4 - 31
1tpp	9.3 ± 6.6	1 - 42
3ptp	9.9 ± 5.1	4 - 40
2ptc	24.7 ± 6.1	10 - 44
1tpa	19.3 ± 5.5	8 - 38

aa	cont	sas	1tpo	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
16I		0	15	16	9-	10	5	22	18
17V		8	12-	14	8-	7	6	22	18
18G		41	16	18	11	6	12	20	17
19G		28	17	17	16	8	7	19	16
20Y		40	9-	15	8-	1-	8	29	24
21T		44	16	18	14	3	8	28	22
22C		12	15	18	11	4	5	26	21
23G		32	15	18	14	6	9	31+	24
24A		51	22+	22+	16	12	7	37+	31+
25N		21	19	29+	14	6	10	34+	27+
26T		54	16	27+	12	5	6	33+	26+
27V		7	16	15	11	5	8	27	20
28P		23	14	11	11	11	11	30	21
29Y		9	13	6-	9	2-	6	18-	12-
30Q		6	12-	10	10	7	8	17-	12-
31V		1	13	13	9	5	5	21	17
32S		0	13	9	10	2-	4-	18-	14
33L		0	11-	9	12	8	9	15-	12-
34N		10	13	10	8-	10	8	15-	11-
37S		25	16	12	14	7	11	24	17
38G		80	14	14	9	9	11	27	18
39Y	74*	52	14	9	9	11	7	21	13-
40H	27*	17	14	9	9	6	4-	16-	12-
41F	60*	15	19	11	14	8	5	16-	11-
42C	14*	6	15	9	8-	5	9	14-	9-
43G		0	15	14	8-	1-	7	16-	10-
44G		0	15	13	12	1-	8	20	14
45S		0	18	11	12	12	7	20	12-
46L		1	14	10	12	11	6	20	15
47I		10	20	18	16	13	11	32+	26+
48N		30	19	11	16	13	13	35+	26+
49S		31	17	15	15	14	13	27	23
50Q		29	18	9	12	6	11	22	16
51W		3	10-	9	6-	2-	8	15-	10-

Appendix D (cont'd)

aa	cont	sas	1tpo	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
52V		0	12-	11	10	2-	6	19	13-
53V		0	14	9	11	5	5	22	14
54S		0	11-	8-	7-	9	4-	19	15
55A		0	11-	7-	5-	2-	5	10-	8-
56A		3	10-	6-	5-	1-	4	17-	14
57H	85*	35	15	10	12	6	5	18-	17
58C	5*	5	13	14	7	5	7	21	16
59Y		52	15	12	13	3	6	24	18
60K	11	36	18	17	16	11	12	28	21
61S		74	26+	32+	24+	29+	18+	32+	26+
62G		72	25+	29+	27+	25+	16+	33+	28+
63I		6	22+	19	20+	23+	12	21	17
64Q		26	16	10	15	8	9	19	19
65V		0	15	11	13	8	10	22	16
66R		22	17	11	11	8	5	20	15
67L		2	15	9	11	6	10	24	16
69G		11	18	11	15	14	9	27	17
70E		10	19	15	14	13	5	26	19
71D		11	13	11	9	3	5	24	17
72N		17	13	12	7	1-	6	24	23
73I		17	17	13	14	12	8	25	22
74N		67	11-	15	7-	2-	5	31+	26+
75V		49	15	15	11	5	7	29	21
76V		71	24+	25+	15	11	6	33+	20
77E		50	25+	36+	15	7	10	37+	27+
78G		67	27+	43+	21+	15	16+	39+	32+
79N		30	25+	37+	14	6	12	36+	28+
80E		20	21+	15	14	18+	11	26	20
81Q		24	16	12	13	9	11	21	15
82F		35	19	12	13	9	8	26	15
83I		13	15	11	13	7	11	18-	14
84S		34	18	16	16	18+	10	22	19
85A		18	15	15	16	12	7	24	17
86S		51	15	13	13	8	6	19	16
87K		32	17	16	15	13	10	26	20
88S		29	17	10	12	12	13	26	21
89I		22	16	9	11	8	7	24	17
90V		31	15	11	11	18+	11	30	24
91H		12	14	12	11	6	6	25	20
92P		69	14	14	12	8	8	28	18
93S		53	13	11	12	7	9	26	22
94Y	2	23	11-	12	7-	2-	10	20	17
95N		40	14	10	13	13	11	23	19
96S	10*	56	10-	12	9	1-	7	25	23
97N	19*	82	14	18	8-	4	7	25	24
98T	11*	49	15	11	10	3	8	24	22
99L	36*	19	16	10	10	5	6	17-	16
100N		11	16	9	11	8	6	19	15

Appendix D (cont'd)

aa	cont	sas	1tpo	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
101N		11	14	7-	9	8	6	30	18
102D	1	0	12-	9	11	4	9	19	18
103I		0	15	7-	8-	7	5	19	13-
104M		0	13	5-	8-	5	8	21	12-
105L		0	11-	8-	10	7	6	25	16
106I		0	12-	7-	9	4	5	21	17
107K		20	16	9	9	2-	6	15-	11-
108L		1	18	11	12	11	9	25	21
109K		62	24+	12	18+	19+	14	30	25+
110S		52	19	14	17	13	12	28	25+
111A		48	17	17	15	10	14	27	24
112A		7	15	14	11	5	13	28	20
113S		64	21+	17	15	22+	11	29	24
114L		37	22+	19	21+	18+	11	39+	28+
115N		50	31+	22+	31+	42+	12	37+	32+
116S		73	29+	30+	25+	27+	20+	39+	34+
117R		47	24+	16	19+	10	28+	32+	25+
118V		2	17	17	14	13	8	25	20
119A		22	16	18	11	3	8	33+	22
120S		38	16	12	13	13	14	29	23
121I		10	16	13	13	13	9	23	19
122S		43	15	15	15	16+	12	24	20
123L		22	17	17	16	11	13	27	23
124P		4	15	15	15	10	11	34+	29+
125T		83	22+	19	23+	16+	18+	43+	34+
127S		56	20	12	20+	16+	14	35+	30+
128C		23	20	14	19+	12	12	29	24
129A		24	17	19	18+	22+	13	33+	29+
130S		62	21+	18	21+	28+	9	29	27+
132A		47	19	13	21+	14	7	24	18
133G		67	15	14	16	3	13	23	16
134T		25	21+	16	20+	15	11	23	17
135Q		62	17	17	15	11	10	27	22
136C		4	14	13	9	10	13	19	15
137L		21	9-	11	7-	5	4-	25	20
138I		1	14	13	10	3	8	25	19
139S		1	13	8-	8-	4	4-	24	17
140G		0	9-	14	9	2-	7	16-	13-
141W		2	19	11	13	7	6	17-	17
142G		0	14	14	10	1	8	22	13-
143N		2	13	17	11	7	8	20	12-
144T		12	14	18	14	9	9	24	19
145K		51	22+	19	19+	14	20+	26	18
146S		41	24+	31+	22+	23+	32+	35+	35+
147S		72	29+	34+	26+	20+	32+	42+	36+
148G		66	27+	34+	28+	38+	40+	44+	38+
149T		71	22+	30+	26+	19+	32+	38+	33+
150S		42	19	21+	15	12	14	29	24

Appendix D (cont'd)

aa	cont	sas	1tpo	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
151Y	44	48	15	14	9	9	14	34+	28+
152P		14	17	21+	17	8	14	23	14
153D		39	19	20	14	12	7	32+	26+
154V		31	15	16	11	5	7	27	16
155L		5	15	13	12	11	9	20	17
156K		20	14	14	10	9	7	27	18
157C		2	15	9	11	7	7	27	20
158L		1	11	13	8-	9	9	18-	14
159K		46	17	12	13	4	7	23	18
160A		0	18	19	14	8	14	24	19
161P		27	15	15	11	6	6	25	16
162I		6	16	14	11	11	9	24	18
163L		17	16	11	15	7	8	32+	25+
164S		36	20	17	14	15	16+	30	22
165D		57	21+	20	22+	22+	24+	32+	24
166S		62	21+	18	15	12	13	28	22
167S		19	18	16	13	2-	9	31+	22
168C		1	16	8	11	10	10	24	19
169K		39	19	10	17	14	14	26	24
170S		63	18	13	15	12	14	27	26
171A		12	18	19	15	4	12	21	16
172Y		1	14	17	9	5	9	22	17
173P		60	20	15	16	25+	14	29	23
174G		99	24+	16	21+	26+	14	33+	30+
175Q	5	48	14	9	11	8	9	34+	27+
176I		10	14	10	14	3	11	33+	23+
177T		29	12-	11	10	4	7	18-	15
178S		59	15	12	15	11	7	28	22
179N		15	12-	11	9	5	7	28	23
180M		3	13	9	11	3	5	29	21
181F		9	15	9	11	5	8	22	16
182C		1	12-	13	10	1-	7	16-	13-
183A		2	15	11	13	7	9	18-	11-
184G		6	13	16	9	5	7	25	22
184Y		32	20	22+	21+	21+	18+	24	18
185L		34	22+	27+	22+	12	14	21	22
186E		84	23+	29+	22+	17+	9	31+	26+
187G		14	26+	22+	20+	21+	24+	26	21
188G		52	20	27+	17	12	14	23	20
188K		42	20	17	17	6	8	26	22
189D	8*	4	16	15	12	6	9	25	17
190S	22*	5	13	15	8-	5	5	24	16
191C	28*	8	16	15	12	4	4-	25	14
192Q	113*	54	15	12	11	3	7	19	12-
193G	31*	20	13	14	9	3	4-	18-	14
194D	18*	0	12-	12	8-	3	9	14-	10-
195S	44*	16	12-	12	12	2-	6	15-	11-

Appendix D (cont'd)

aa	cont	sas	1tpo	3ptn	3ptb	1tpp	3ptp	2ptc	1tpa
196G		0	9-	5	4-	1-	8	16-	14
197G		1	14	13	5-	2-	4-	19	14
198P		1	16	10	9	7	6	24	15
199V		0	13	11	10	11	8	19	15
200V		9	12-	8-	8-	2-	7	18-	19
201C		4	14	9	9	4	8	21	17
202S		79	19	14	19+	22+	18+	37+	31+
203G		53	17	14	17	12	11	31+	22
204K		34	14	7-	12	6	11	22	17
209L		0	15	12	13	15	5	28	22
210Q		6	13	9	9	5	10	18+	14
211G		0	12-	7-	7-	x	4-	16-	14
212I		0	13	7-	12	4-	4-	23	20
213V	6*	4	12-	6-	7	7-	4-	16-	10-
214S	31*	3	14	7-	7	1-	9	16-	11-
215W	75*	18	15	13	11	11	9	20	11-
216G	31*	31	20	16	17	5	14	25	20
217S		41	16	20	16	8	14	24	22
219G	6*	59	15	25+	14	8	14	19	14
220C	8*	6	14	18	12	12	13	25	22
221A		8	15	21+	11	5	11	22	14
221Q		40	21+	26+	19	15	8	24	20
222K		72	17	19	15	14	20+	25	20
223N		44	19	32+	19	13	13	29	24
224K		31	16	16	15	11	11	28	21
225P		4	18	17	20	13	14	27	19
226G	4*	1	10-	15	11	1-	9	20	17
227V	2*	0	12-	8-	9	5	9	14-	14
228Y	1	1	11-	7-	8	1-	6	22	15
229T		1	11-	10	7	11	4-	18-	14
230K		30	10-	6-	8	x	7	24	17
231V		0	13	9	9	12	7	23	19
232C		15	16	16	11	4	8	25	18
233N		43	11-	8-	9	7	11	19	13-
234Y		42	15	9	7	6	7	20	12-
235V		17	16	9	13	5	5	25	20
236S		65	16	14	16	6	16+	22	20
237W		18	15	10	12	7	9	25	19
238I		1	14	9	8	7	5	23	20
239K		49	16	14	11	14	12	23	19
240Q		62	17	14	16	16+	9	23	20
241T		10	15	11	12	11	10	24	20
242I		23	16	14	13	10	13	20	19
243A		68	21+	13	17	8	14	26	21
244S		56	22+	13	19	13	13	18-	15
245N		31	20	11	16	10	11	24	21

Appendix E

'Substrates' v. 1tpo (NO CONSTRAINTS)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.39	.46	.42	.37	.55	.61	.58	.53	.16	.14	.15	.54	.50	.52	.46
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.52	.37(18)	1.2	1.6	0.6	0.2	2.0	1.7	0.3	0.2	1.7	1.2	0.8	0.7	1.2	1.0	0.7
02	.67	.45(18)	1.5	1.4	0.3	0.3	2.3	1.9	2.6	0.3	1.7	1.6	0.9	0.4	3.9	0.9	0.2
03	.51	.38(17)	1.0	1.4	0.5	0.2	1.7	1.5	1.2	0.3	1.6	1.2	0.7	1.1	1.6	0.8	0.7
04	.36	.18(26)	1.2	0.2	0.4	0.9	1.0	0.8	2.3	0.6	1.9	1.2	1.1	0.1	1.7	0.8	0.1
05	.60	.45(18)	1.3	1.3	0.4	1.2	1.8	1.9	2.7	0.8	2.6	1.7	1.2	0.3	1.3	0.9	0.3
06	.64	.56(8)	0.9	1.5	0.8	0.6	2.2	2.2	1.7	0.6	1.6	1.2	0.8	1.0	1.3	0.7	0.4
07	.58	.48(10)	0.9	1.2	0.7	0.2	2.2	2.4	1.7	0.5	1.8	1.3	0.8	0.5	0.5	0.7	0.4
08	.66	.55(11)	1.1	1.2	0.4	1.0	2.2	2.5	1.4	1.0	2.3	2.0	1.3	0.4	1.3	1.0	0.5
09	.60	.50(11)	1.3	1.1	0.3	0.3	2.1	1.8	1.8	0.8	2.2	1.5	1.1	0.4	1.4	0.9	0.4
10	.52	.37(18)	0.9	1.0	0.4	0.2	1.8	1.9	0.7	0.3	1.9	1.5	1.1	0.7	1.3	1.0	0.7
11	.42	.26(22)	0.9	0.6	0.5	0.4	1.6	1.1	0.7	0.6	1.9	1.2	0.9	0.7	0.4	0.8	0.6
12	.44	.30(21)	1.4	0.8	0.7	0.2	1.9	1.4	2.0	0.2	1.5	1.2	0.8	0.3	1.3	0.8	0.1
13	.53	.37(18)	1.2	1.3	0.5	0.3	1.8	1.3	2.3	0.5	1.8	1.4	1.0	1.0	0.8	0.7	0.7
14	.50	.37(18)	1.1	1.4	0.5	0.3	1.5	1.6	0.8	0.5	1.7	1.2	0.9	0.9	1.2	1.0	0.5
15	.49	.38(15)	1.1	0.9	0.6	0.2	1.5	1.0	0.9	0.2	1.4	1.2	0.8	0.7	1.4	1.0	0.7
16	.51	.38(17)	1.2	1.3	0.5	0.2	1.6	1.8	0.8	0.4	1.7	1.3	0.9	1.0	1.3	0.9	0.7
17	.45	.31(25)	1.2	1.7	0.6	0.2	1.4	1.5	1.4	0.8	1.2	1.1	0.8	1.3	1.3	0.7	0.3
18	.46	.31(26)	1.5	0.8	0.6	0.2	1.6	1.6	0.8	0.4	1.5	1.1	0.8	1.1	1.4	0.8	0.3
19	.42	.25(22)	1.0	0.9	0.5	0.2	1.5	1.7	0.5	0.5	1.6	0.9	0.5	0.7	1.4	1.0	0.4
20	.46	.30(20)	1.3	1.2	0.5	1.2	1.7	1.8	0.5	0.5	1.5	1.0	0.6	0.6	1.4	1.0	0.6

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.45	.29(21)	1.2	1.3	0.5	0.2	1.8	1.5	0.2	0.4	1.6	1.1	0.7	0.7	1.1	1.0	0.6
02	.74	.49(22)	1.5	1.5	0.5	0.5	2.3	1.9	2.7	0.4	1.8	1.6	0.9	0.4	4.2	0.9	0.3
03	.49	.36(17)	1.0	1.4	0.4	0.3	1.7	1.5	1.5	0.5	1.5	1.2	0.7	1.1	1.6	0.8	0.5
04	.46	.32(19)	1.2	1.1	0.3	1.4	1.4	1.7	0.7	0.6	1.9	1.4	1.1	0.2	1.4	1.0	0.2
05	.61	.47(16)	1.2	1.3	0.4	1.2	1.8	1.9	2.5	1.6	2.5	1.8	1.2	0.3	1.3	0.9	0.3
06	.46	.38(10)	0.8	1.0	0.8	0.3	1.8	1.7	1.2	0.7	1.8	1.0	0.8	0.5	0.8	0.6	0.2
07	.58	.48(9)	0.9	1.2	0.6	0.5	2.2	2.4	1.6	0.5	1.8	1.2	0.8	1.2	0.5	0.6	0.4
08	.61	.49(13)	1.1	1.1	0.3	1.0	2.1	2.4	1.2	0.8	2.2	1.7	1.0	0.4	1.3	1.0	0.4
09	.64	.51(17)	1.3	1.4	0.5	0.6	2.0	1.6	2.4	0.6	2.0	1.6	1.1	0.4	1.4	0.9	0.3
10	.54	.39(18)	1.2	1.3	0.3	0.8	1.8	2.0	0.6	0.3	1.9	1.5	1.1	0.7	1.3	1.0	0.7
11	.45	.28(20)	1.0	0.7	0.4	0.4	1.9	1.7	0.6	0.3	1.8	1.2	1.0	0.8	1.0	0.7	0.4
12	.55	.44(15)	1.3	1.5	0.6	0.4	2.2	1.8	1.5	0.7	1.8	1.4	0.9	0.4	1.1	0.7	0.2
13	.47	.33(18)	1.2	1.0	0.5	0.4	1.8	1.4	0.8	0.5	1.7	1.2	0.9	0.7	1.3	0.7	0.7
14	.50	.37(19)	1.1	1.4	0.4	0.4	1.5	1.6	0.9	0.5	1.7	1.2	0.9	0.9	1.2	1.0	0.5
15	.73	.54(11)	1.4	1.1	0.5	0.3	2.1	1.8	2.8	0.4	1.9	1.5	0.9	1.1	5.1	0.9	0.2
16	.47	.32(19)	1.3	1.1	0.5	0.3	1.5	1.7	0.7	0.5	1.6	1.2	0.8	0.9	1.2	0.9	0.6
17	.44	.29(26)	1.2	1.1	0.5	0.3	1.4	1.5	1.4	0.7	1.2	1.1	0.7	1.3	1.3	0.7	0.2
18	.47	.31(24)	1.5	0.8	0.5	0.2	1.8	1.3	1.7	0.1	1.4	1.2	0.8	1.3	1.4	0.8	0.3
19	.50	.36(19)	1.0	1.2	0.5	0.3	1.9	2.0	0.8	0.4	1.7	1.2	0.7	0.8	1.6	1.0	0.7
20	.53	.38(17)	1.2	1.4	0.5	0.3	1.8	1.9	0.4	0.3	1.5	1.1	0.7	0.8	1.5	1.0	0.7

Appendix E (cont'd)

'Enzymes' v. 2ptc (NO CONSTRAINTS)

	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B	.52	.45	.45	.42	.45	.36	.50	.66	.49	.44	.45	.45	.50
1tpo-2ptc	.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.57	.45(16)	1.1	1.1	0.9	0.8	0.4	1.0	1.0	0.8	1.1	1.8	0.7	1.2	1.1
02	.61	.57(9)	1.0	1.0	0.6	0.6	0.3	0.5	1.1	0.7	1.3	1.4	0.6	1.1	1.2
03	.56	.39(28)	1.3	1.1	1.0	0.7	0.3	1.2	1.1	1.0	1.6	1.6	0.8	1.0	1.0
04	.37	.22(30)	0.5	0.5	0.6	0.9	0.5	0.5	0.5	0.4	0.9	1.5	0.8	1.6	1.4
05	.60	.54(8)	1.1	1.1	0.7	0.8	0.4	0.8	0.5	0.5	0.8	1.6	0.7	1.0	1.1
06	.69	.59(12)	0.7	0.7	0.5	0.4	0.5	1.0	1.1	0.8	1.4	1.7	0.8	0.8	1.0
07	.65	.50(19)	0.5	0.8	0.5	0.5	0.5	0.9	1.3	0.6	1.5	1.6	0.8	0.7	0.9
08	.68	.63(5)	1.1	1.0	0.7	0.6	0.3	0.8	1.0	1.0	1.5	1.5	0.7	1.3	1.3
09	.64	.56(7)	1.1	1.0	0.7	0.6	0.3	1.2	0.7	0.5	0.9	1.9	0.7	1.1	1.1
10	.54	.44(19)	1.2	1.1	0.7	0.8	0.3	1.1	0.9	0.6	1.1	1.5	1.6	1.2	1.1
11	.48	.28(35)	0.9	0.9	0.8	0.8	0.4	1.1	0.8	0.6	1.2	1.8	0.7	1.4	1.5
12	.47	.38(14)	0.8	0.8	0.6	0.8	0.4	0.8	0.7	0.4	0.9	1.6	0.7	1.2	1.1
13	.56	.43(22)	1.0	1.0	0.8	0.9	0.4	0.5	1.1	0.9	1.3	1.5	0.6	1.1	1.0
14	.59	.43(21)	1.1	1.1	0.8	0.8	0.4	1.1	1.0	0.8	1.4	1.8	0.7	1.1	1.2
15	.56	.46(16)	1.4	1.2	1.1	1.0	0.9	1.0	1.1	0.8	1.2	1.5	0.8	1.2	1.1
16	.44	.29(30)	0.8	1.1	1.0	1.0	0.9	0.9	0.8	0.9	1.4	1.6	0.8	1.1	1.1
17	.50	.35(22)	0.9	1.1	0.7	0.8	0.6	1.0	1.1	0.9	1.2	1.6	0.7	1.0	1.0
18	.51	.37(25)	0.8	1.0	0.7	0.8	0.4	1.1	1.1	0.8	1.3	1.7	0.6	0.9	0.9
19	.44	.27(34)	0.7	1.1	0.8	0.7	0.3	1.1	0.8	0.8	1.0	1.7	0.7	1.0	1.2
20	.49	.32(30)	1.0	1.1	0.8	0.8	0.4	1.2	1.0	0.9	1.1	1.7	0.7	0.9	1.1

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.44	.25(36)	0.9	1.0	0.8	0.8	0.4	1.0	0.8	0.7	0.9	1.9	0.8	1.3	1.2
02	.52	.42(15)	0.8	0.9	0.6	0.7	0.3	0.8	1.0	0.8	1.1	1.5	0.7	1.1	1.2
03	.53	.33(32)	1.2	1.0	0.9	0.7	0.3	1.2	1.0	1.1	1.5	1.9	0.8	1.1	1.1
04	.69	.63(7)	1.2	1.0	0.5	0.5	0.3	0.4	0.8	0.5	1.0	1.5	0.6	1.4	2.0
05	.66	.58(9)	1.2	1.1	0.6	0.7	0.5	1.0	0.6	0.5	0.9	1.6	0.7	0.9	1.0
06	.53	.42(17)	0.7	0.8	0.5	0.7	0.3	1.2	0.9	0.7	1.0	1.7	0.8	1.1	1.1
07	.70	.59(14)	0.6	0.6	0.5	0.4	0.6	0.7	1.4	0.6	1.5	1.5	0.8	0.8	0.9
08	.60	.53(10)	1.1	1.0	0.7	0.6	0.3	0.8	0.9	0.7	1.3	1.6	0.7	1.3	1.2
09	.68	.59(8)	1.2	1.0	0.7	0.5	0.3	0.8	0.7	0.4	0.9	2.0	0.7	1.3	1.2
10	.46	.34(23)	1.0	0.9	0.7	0.8	0.3	1.2	0.7	0.6	1.0	1.5	0.6	1.2	1.0
11	.60	.45(18)	0.9	0.8	0.6	0.9	0.4	1.1	0.8	0.6	1.4	1.2	0.7	1.2	1.0
12	.65	.57(9)	1.2	0.9	0.6	0.7	0.4	0.4	0.9	0.5	1.0	1.7	0.7	1.2	1.1
13	.57	.41(25)	1.0	1.0	0.7	0.8	0.4	1.0	1.1	0.9	1.6	1.7	0.6	1.1	1.1
14	.55	.39(22)	1.0	1.0	0.8	0.8	0.4	1.2	0.9	0.8	1.3	1.9	0.7	1.0	1.2
15	.57	.41(25)	1.3	1.1	1.0	1.0	0.6	0.8	1.3	0.8	1.4	1.6	0.7	1.1	1.1
16	.46	.27(36)	0.8	1.0	0.7	0.8	0.4	1.2	0.9	0.9	1.2	1.9	0.8	0.8	1.0
17	.53	.36(27)	0.9	1.1	0.8	1.0	0.4	1.2	1.0	0.7	1.4	1.8	0.8	0.8	1.0
18	.52	.38(24)	0.8	1.0	0.7	0.8	0.4	1.2	1.2	0.8	1.3	1.7	0.6	0.9	1.1
19	.47	.31(32)	0.8	1.1	0.8	0.7	0.3	1.2	0.9	0.8	1.1	1.7	0.7	1.1	1.2
20	.55	.40(28)	1.0	1.2	0.8	0.7	0.3	1.1	1.1	1.1	1.3	1.7	0.7	1.0	1.0

Appendix E (cont'd)

'Substrates' v. 1tpo (N CONSTRAINED)

			17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.39	.46	.42	.37	.55	.61	.58	.53	.16	.14	.15	.54	.50	.52	.46
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.68	.38(28)	1.9	1.6	0.6	0.2	2.5	3.8	1.3	0.9	3.0	1.8	1.3	0.5	1.1	1.5	0.8
02	.63	.34(29)	2.0	1.5	0.5	0.3	2.5	3.1	0.7	0.9	3.0	1.7	1.5	0.7	1.8	1.1	0.9
03	.62	.33(27)	2.0	2.0	0.5	0.2	2.2	3.0	1.5	1.3	2.2	1.7	1.1	0.6	1.6	1.2	0.7
04	.56	.30(25)	1.6	1.0	0.3	1.1	2.1	3.1	1.1	1.4	3.0	1.6	1.6	0.3	1.2	1.1	0.2
05	.71	.47(26)	2.1	1.0	0.5	1.2	2.6	2.8	1.4	1.6	3.4	2.4	1.9	0.2	1.4	1.2	0.4
06	.87	.59(23)	2.0	1.1	0.8	0.2	3.3	3.6	1.5	1.1	4.4	2.5	1.4	1.2	1.9	0.8	0.1
07	.62	.41(26)	1.3	1.1	1.1	0.2	2.8	3.1	1.0	0.7	1.8	1.9	1.0	0.8	1.2	0.8	0.1
08	.90	.58(25)	1.9	1.8	0.6	0.3	2.2	5.3	3.3	1.5	4.0	3.0	1.8	0.6	2.0	1.7	1.0
09	.76	.49(21)	2.1	1.0	0.4	0.5	2.3	4.1	3.6	0.7	3.4	2.2	1.7	0.5	1.6	1.4	0.5
10	.69	.40(23)	2.1	1.4	0.4	0.9	2.5	3.6	1.6	1.2	2.9	2.1	1.7	0.7	1.3	1.3	0.8
11	.66	.40(23)	1.9	1.1	0.4	0.6	2.5	3.3	0.9	1.0	3.1	1.9	1.6	0.6	1.1	1.2	0.7
12	.79	.53(25)	2.1	1.5	0.8	0.2	3.4	3.2	1.4	0.7	3.2	2.3	1.5	0.7	1.7	1.0	0.1
13	.83	.50(35)	2.2	1.5	0.5	0.4	3.4	3.4	1.6	1.5	2.6	2.4	2.0	0.8	1.9	1.0	0.2
14	.70	.39(25)	1.9	1.9	0.4	0.3	2.4	3.6	2.4	1.4	3.1	2.2	1.5	0.8	1.5	1.4	0.7
15	.64	.38(26)	1.7	1.1	0.6	0.2	2.8	3.2	1.0	0.3	2.6	1.6	1.3	1.2	2.1	1.2	0.7
16	.72	.40(32)	2.2	2.3	0.5	0.3	3.1	3.2	1.1	1.0	2.3	2.0	1.5	0.8	1.5	1.3	0.9
17	.71	.41(35)	2.1	1.4	0.6	0.3	3.1	3.5	1.6	1.6	2.3	2.1	1.4	1.4	1.7	1.5	0.5
18	.90	.57(29)	2.3	1.4	0.5	0.3	3.4	3.7	3.0	1.9	3.0	2.8	1.5	1.5	2.6	1.4	0.4
19	.43	.17(32)	1.4	0.6	0.3	0.2	1.6	2.2	2.3	0.5	1.9	0.9	0.4	0.5	1.1	1.1	0.3
20	.70	.39(25)	2.1	2.0	0.5	0.2	2.6	3.7	1.3	0.7	2.2	1.8	1.1	1.0	1.3	1.4	0.8

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.61	.33(29)	1.8	1.2	0.6	0.3	2.6	3.3	1.0	0.8	2.9	1.6	1.2	0.6	1.1	1.4	0.7
02	.67	.35(35)	2.0	1.6	0.4	0.5	2.5	3.4	0.9	0.9	3.0	1.7	1.5	1.3	1.9	1.1	1.0
03	.72	.39(30)	1.9	2.2	0.4	0.3	2.6	3.6	1.5	1.5	2.9	2.1	1.2	1.0	1.8	1.3	0.8
04	.88	.60(23)	1.6	2.1	0.6	1.8	2.3	4.9	2.2	1.4	3.9	2.5	1.8	1.0	1.9	1.2	0.5
05	.85	.60(18)	1.9	1.1	0.7	1.3	3.1	3.7	2.8	1.9	3.8	2.9	1.7	0.5	1.7	1.1	0.6
06	.81	.55(23)	2.0	1.0	0.7	0.7	3.3	3.5	1.4	0.8	4.0	2.3	1.4	0.9	1.6	0.8	0.3
07	.78	.65(10)	1.3	1.1	0.8	0.7	3.2	3.3	2.0	1.3	2.4	2.1	0.8	1.1	1.4	0.6	0.5
08	.78	.49(23)	1.9	1.4	0.3	0.8	2.3	4.5	3.5	1.1	3.3	2.3	1.6	0.8	1.7	1.6	0.9
09	.86	.56(24)	2.1	1.2	0.7	0.7	2.3	3.8	4.7	0.9	3.8	2.5	1.7	0.5	1.8	1.4	0.4
10	.44	.22(24)	1.7	0.4	0.4	0.5	1.7	1.9	0.8	1.3	2.5	1.4	1.2	0.5	1.0	1.0	0.4
11	.67	.39(22)	1.8	1.0	0.4	0.6	2.6	3.5	2.8	0.8	3.0	1.9	1.6	0.6	1.2	1.2	0.7
12	.88	.64(21)	2.2	1.7	0.8	0.8	3.5	3.6	1.8	1.2	3.3	2.4	1.4	0.9	1.8	1.0	0.2
13	.67	.36(32)	2.1	1.5	0.5	0.5	2.7	2.8	0.8	0.4	2.5	1.9	1.6	1.4	0.9	1.2	0.6
14	.70	.39(26)	1.9	1.8	0.4	0.4	2.4	3.6	2.3	1.4	3.1	2.2	1.5	0.8	1.1	1.3	0.9
15	.66	.40(24)	1.7	1.2	0.5	0.4	2.8	3.5	1.2	0.2	2.6	1.7	1.3	0.8	1.1	1.3	1.0
16	.70	.38(36)	2.2	1.6	0.4	0.4	3.1	3.1	1.2	1.0	2.3	2.0	1.5	1.0	1.6	1.3	1.0
17	.67	.37(34)	2.1	1.4	0.5	0.4	2.8	3.3	1.3	1.3	2.1	1.8	1.3	1.4	1.6	1.5	0.5
18	.86	.59(24)	2.1	0.9	0.4	0.7	3.4	3.5	1.7	1.6	3.4	2.6	1.5	1.3	2.4	1.4	0.4
19	.60	.31(28)	1.8	1.1	0.5	0.3	2.4	2.7	2.7	0.9	2.3	1.8	1.0	0.6	1.6	1.4	0.5
20	.73	.40(26)	2.0	2.0	0.4	0.4	2.6	4.1	2.1	1.4	3.1	2.2	1.1	0.7	1.4	1.2	0.9

Appendix E (cont'd)

'Enzymes' v. 2ptc (N CONSTRAINED)

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B			.52	.45	.45	.42	.45	.36	.50	.66	.49	.44	.45	.45	.50
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24
01	.14	.12(7)	0.2	0.3	0.1	0.2	0.1	0.4	0.1	0.2	0.1	0.3	0.2	0.3	0.0
02	.14	.12(8)	0.1	0.2	0.0	0.3	0.1	0.4	0.2	0.2	0.2	0.4	0.2	0.3	0.0
03	.14	.12(9)	0.1	0.3	0.0	0.3	0.1	0.4	0.1	0.2	0.2	0.4	0.1	0.0	0.1
04	.15	.11(7)	0.1	0.1	0.1	0.3	0.1	0.6	0.1	0.1	0.3	0.4	0.2	0.3	0.8
05	.16	.11(15)	0.2	0.1	0.1	0.2	0.1	0.3	0.1	0.2	0.1	0.9	0.8	0.1	0.0
06	.13	.11(8)	0.4	0.1	0.0	0.1	0.1	0.3	0.1	0.1	0.1	0.4	0.2	0.1	0.1
07	.13	.12(5)	0.2	0.1	0.0	0.1	0.1	0.4	0.1	0.2	0.4	0.5	0.2	0.1	0.1
08	.14	.11(13)	0.0	0.1	0.0	0.1	0.1	0.1	0.1	0.2	0.3	0.1	0.0	0.2	0.1
09	.14	.11(11)	0.0	0.1	0.0	0.2	0.1	0.0	0.1	0.2	0.1	0.3	0.1	0.2	0.0
10	.13	.12(9)	0.1	0.1	0.1	0.2	0.1	0.3	0.1	0.2	0.1	0.4	0.2	0.3	0.1
11	.14	.12(10)	0.1	0.1	0.1	0.1	0.1	0.5	0.1	0.2	0.1	0.4	0.2	0.3	0.0
12	.13	.12(5)	0.2	0.1	0.0	0.1	0.1	0.3	0.1	0.1	0.0	0.4	0.2	0.2	0.1
13	.13	.13(5)	0.4	0.1	0.1	0.1	0.1	0.3	0.0	0.1	0.2	0.4	0.2	0.1	0.2
14	.14	.12(5)	0.2	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.3	0.1	0.1	0.1
15	.14	.12(9)	0.1	0.3	0.2	0.2	0.1	0.5	0.1	0.2	0.2	0.4	0.1	0.3	0.1
16	.13	.12(9)	0.1	0.2	0.1	0.1	0.1	0.3	0.1	0.2	0.1	0.4	0.1	0.2	0.2
17	.13	.12(7)	0.2	0.1	0.1	0.1	0.1	0.3	0.1	0.2	0.1	0.4	0.2	0.0	0.1
18	.13	.11(9)	0.4	0.0	0.1	0.1	0.1	0.1	0.0	0.2	0.3	0.4	0.1	0.1	0.1
19	.14	.11(10)	0.1	0.4	0.2	0.2	0.0	0.4	0.1	0.2	0.2	0.7	0.1	0.2	0.2
20	.14	.12(9)	0.2	0.3	0.1	0.2	0.1	0.3	0.1	0.2	0.1	0.3	0.2	0.4	0.2

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.14	.12(10)	0.2	0.3	0.1	0.2	0.1	0.5	0.1	0.2	0.2	0.5	0.2	0.3	0.0
02	.14	.12(8)	0.1	0.2	0.0	0.2	0.1	0.4	0.2	0.2	0.1	0.4	0.2	0.1	0.0
03	.13	.12(6)	0.2	0.2	0.0	0.2	0.1	0.2	0.1	0.2	0.2	0.4	0.2	0.1	0.0
04	.15	.12(8)	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.0	0.2	0.5
05	.15	.11(11)	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.3	0.1	0.1	0.1
06	.13	.12(7)	0.3	0.1	0.0	0.1	0.1	0.3	0.1	0.1	0.1	0.4	0.2	0.1	0.1
07	.13	.12(8)	0.4	0.1	0.0	0.1	0.1	0.1	0.1	0.2	0.4	0.4	0.2	0.1	0.1
08	.13	.11(12)	0.0	0.1	0.0	0.2	0.1	0.1	0.1	0.2	0.3	0.2	0.1	0.2	0.1
09	.14	.11(11)	0.1	0.1	0.0	0.2	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.1
10	.15	.11(11)	0.1	0.2	0.1	0.4	0.1	0.6	0.1	0.2	0.1	0.8	0.8	0.3	0.3
11	.14	.12(10)	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.2	0.3	0.3	0.1	0.2	0.1
12	.13	.11(8)	0.4	0.1	0.0	0.1	0.1	0.2	0.1	0.2	0.2	0.4	0.2	0.1	0.0
13	.14	.12(8)	0.1	0.2	0.1	0.1	0.1	0.5	0.1	0.2	0.2	0.4	0.1	0.2	0.0
14	.13	.12(6)	0.2	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.3	0.1	0.1	0.1
15	.14	.12(9)	0.1	0.2	0.2	0.2	0.1	0.4	0.1	0.2	0.2	0.3	0.1	0.2	0.1
16	.13	.12(9)	0.1	0.2	0.2	0.1	0.1	0.3	0.1	0.2	0.2	0.4	0.1	0.2	0.2
17	.13	.11(7)	0.1	0.2	0.1	0.1	0.1	0.3	0.1	0.2	0.1	0.4	0.1	0.0	0.1
18	.13	.12(6)	0.4	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.4	0.2	0.1	0.1
19	.14	.12(12)	0.2	0.3	0.0	0.4	0.1	0.2	0.1	0.2	0.1	0.5	0.1	0.3	0.3
20	.13	.12(4)	0.1	0.3	0.2	0.2	0.1	0.2	0.1	0.2	0.1	0.3	0.1	0.1	0.1

Appendix E (cont'd)

'Enzyme' v. 2ptc (N,C CONSTRAINED)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.39	.46	.42	.37	.55	.61	.58	.53	.16	.14	.15	.54	.50	.52	.46
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.69	.38(27)	1.8	1.8	0.6	0.2	2.6	3.7	3.1	0.8	2.2	1.8	1.2	0.8	1.4	1.4	0.7
02	.82	.45(34)	2.4	1.6	0.4	0.2	2.9	3.7	2.7	1.1	2.5	2.0	1.7	1.0	2.4	1.3	0.3
03	.65	.30(32)	1.8	2.0	0.5	0.2	2.5	3.4	0.8	0.9	2.7	1.6	1.1	0.6	1.1	1.2	0.8
04	.79	.50(21)	2.0	0.9	0.4	0.7	3.3	4.2	2.5	2.1	3.8	3.0	1.7	0.3	1.1	1.5	0.3
05	.68	.40(23)	1.9	0.6	0.1	0.4	3.0	3.4	2.7	1.0	3.1	2.4	1.4	0.1	1.1	0.9	0.3
06	.81	.62(15)	2.0	1.9	1.0	0.8	3.5	3.9	1.5	1.3	2.5	2.2	1.6	0.7	1.5	1.0	0.5
07	.73	.48(24)	2.0	1.6	1.0	0.8	2.4	3.7	1.3	1.2	2.4	2.2	1.6	0.6	1.4	0.9	0.4
08	.83	.53(25)	2.1	2.2	0.6	1.0	3.2	3.8	3.6	1.1	3.2	2.3	1.8	0.5	2.1	1.5	0.7
09	.67	.39(24)	2.0	0.7	0.5	0.4	2.8	4.2	1.7	1.2	2.5	2.0	1.6	0.4	1.7	1.3	0.4
10	.71	.42(22)	2.0	1.4	0.4	0.8	2.8	4.1	1.7	1.1	2.8	1.9	1.7	0.8	1.3	1.2	0.8
11	.54	.26(29)	1.7	0.4	0.5	0.4	2.5	3.1	1.0	1.3	2.2	1.8	1.5	0.7	0.9	1.1	0.7
12	.77	.49(28)	0.2	1.4	0.6	0.2	3.1	3.4	1.3	0.9	3.3	2.2	1.5	0.6	1.9	0.9	0.1
13	.75	.40(33)	2.0	1.8	0.5	0.3	0.4	2.8	3.7	2.4	2.1	2.5	1.8	0.7	1.6	1.0	0.5
14	.46	.18(36)	1.7	0.8	0.4	0.2	2.1	2.7	1.3	1.1	2.0	1.2	0.9	0.6	0.9	1.2	0.5
15	.69	.40(34)	2.2	1.3	0.6	0.2	3.1	3.2	1.3	1.5	2.6	2.1	1.4	0.8	2.1	1.2	0.3
16	.73	.39(34)	2.1	1.8	0.5	0.2	2.8	3.5	1.0	1.0	2.7	2.2	1.6	0.8	1.5	1.3	1.0
17	.78	.50(29)	2.2	1.4	0.5	0.2	3.3	3.6	2.3	2.0	2.1	2.0	1.3	1.4	2.0	1.5	0.5
18	.67	.35(34)	2.2	1.4	0.6	0.2	2.8	3.5	1.7	1.3	1.8	1.7	1.3	1.7	2.0	1.3	0.6
19	.70	.39(27)	1.9	1.7	0.6	0.2	2.7	3.2	2.7	1.6	2.7	2.5	1.2	0.5	1.5	1.5	0.7
20	.71	.39(33)	2.0	1.7	0.5	0.2	3.1	3.4	1.4	0.7	3.1	1.8	1.2	0.6	1.5	1.5	0.9

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.70	.38(28)	1.8	1.8	0.5	0.3	2.6	3.6	2.9	0.9	2.8	1.9	1.2	0.5	1.2	1.4	0.8
02	.65	.32(33)	2.0	1.6	0.4	0.3	2.7	3.0	1.7	1.5	2.3	1.9	1.5	0.8	1.6	1.1	0.9
03	.75	.39(32)	1.7	2.0	0.4	0.3	2.8	3.7	2.5	1.8	2.7	2.0	1.2	1.0	1.8	1.3	1.0
04	.91	.60(22)	1.7	2.0	0.5	1.8	3.2	3.2	3.9	1.2	3.6	3.2	2.4	0.3	1.9	1.3	0.4
05	.80	.54(22)	2.1	1.1	0.6	1.3	3.2	3.7	2.6	1.3	3.2	2.5	2.1	0.2	1.5	1.0	0.2
06	.70	.48(20)	1.7	1.0	0.8	0.5	3.2	3.4	1.9	0.7	2.3	2.2	1.1	0.8	1.3	0.9	0.2
07	.88	.71(11)	1.4	1.8	0.7	0.6	3.4	3.5	2.1	1.8	2.8	2.5	1.0	1.5	1.5	0.6	0.9
08	.83	.53(25)	2.1	2.2	0.5	0.9	3.2	3.8	3.6	1.2	3.2	2.2	1.8	0.5	2.1	1.5	0.7
09	.76	.47(26)	2.2	1.2	0.4	0.7	3.1	3.7	3.3	1.0	2.8	2.0	1.6	0.4	1.9	1.3	0.5
10	.72	.42(23)	2.0	1.4	0.3	1.0	2.8	4.1	1.1	1.1	2.8	1.9	1.7	0.8	1.2	1.2	0.8
11	.65	.39(20)	1.9	0.8	0.5	0.5	2.8	3.7	1.3	1.1	2.3	2.1	1.7	0.8	1.0	1.1	0.7
12	.76	.50(28)	2.2	1.3	0.6	0.5	3.0	3.4	1.3	1.0	3.2	2.1	1.5	0.6	1.9	0.9	0.1
13	.65	.32(34)	1.9	1.8	0.5	0.4	2.7	3.4	1.1	1.4	2.4	2.2	1.7	0.7	1.5	0.9	0.9
14	.47	.33(19)	1.1	1.3	0.4	0.3	1.4	1.5	0.9	0.5	1.7	1.2	0.8	0.9	1.1	1.0	0.5
15	.84	.60(20)	2.3	1.6	0.5	0.5	3.4	3.6	2.1	1.9	2.7	2.3	1.3	0.8	2.3	1.0	0.2
16	.58	.26(33)	1.9	1.7	0.5	0.2	2.6	3.1	0.8	1.3	2.3	1.7	1.1	0.7	1.3	1.1	0.7
17	.89	.64(23)	2.3	1.8	0.6	0.8	3.4	3.5	2.6	2.4	2.4	2.2	1.4	1.7	2.0	1.2	0.5
18	.70	.37(34)	2.2	1.4	0.5	0.3	2.8	3.6	2.1	1.6	1.9	1.8	1.3	1.7	2.1	1.3	0.6
19	.48	.22(30)	1.5	0.4	0.4	0.2	2.4	2.5	1.5	1.2	2.0	1.3	0.7	0.5	1.2	1.3	0.4
20	.64	.32(35)	1.9	1.4	0.5	0.3	3.0	3.4	1.3	0.5	2.8	1.6	1.0	0.5	1.5	1.4	0.8

Appendix E (cont'd)

'Enzyme' v. 2ptc (N,C CONSTRAINED)

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B			.52	.45	.45	.42	.45	.36	.50	.66	.49	.44	.45	.45	.50
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24
01	.09	.08(6)	0.2	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.3	0.0	0.2	0.2
02	.10	.08(10)	0.3	0.1	0.0	0.1	0.1	0.0	0.1	0.1	0.1	0.3	0.0	0.2	0.1
03	.10	.08(7)	0.1	0.2	0.0	0.2	0.0	0.4	0.1	0.1	0.2	0.3	0.0	0.2	0.2
04	.46	.45(1)	0.6	0.5	0.3	0.4	0.5	0.5	0.5	0.2	0.5	0.7	0.5	0.2	0.2
05	.46	.45(1)	0.7	0.5	0.3	0.2	0.5	0.5	0.5	0.2	0.5	0.6	0.5	0.3	0.2
06	.46	.45(2)	0.7	0.5	0.3	0.3	0.5	0.4	0.5	0.2	0.5	0.7	0.5	0.4	0.1
07	.46	.45(2)	0.7	0.4	0.1	0.2	0.5	0.5	0.5	0.3	0.4	0.7	0.5	0.3	0.2
08	.09	.08(9)	0.1	0.1	0.1	0.0	0.0	0.1	0.0	0.1	0.1	0.3	0.0	0.1	0.1
09	.09	.08(9)	0.2	0.1	0.1	0.1	0.0	0.4	0.0	0.1	0.0	0.2	0.0	0.2	0.2
10	.09	.08(7)	0.2	0.1	0.0	0.1	0.0	0.2	0.0	0.1	0.1	0.2	0.0	0.2	0.1
11	.10	.08(9)	0.1	0.1	0.0	0.2	0.0	0.6	0.0	0.1	0.2	0.3	0.1	0.2	0.3
12	.09	.08(7)	0.2	0.0	0.0	0.0	0.0	0.4	0.0	0.1	0.1	0.3	0.0	0.2	0.1
13	.09	.08(6)	0.2	0.1	0.0	0.1	0.0	0.2	0.1	0.1	0.1	0.3	0.0	0.0	0.2
14	.10	.07(8)	0.1	0.2	0.0	0.2	0.0	0.4	0.1	0.1	0.0	0.4	0.0	0.2	0.4
15	.09	.08(4)	0.2	0.1	0.0	0.1	0.0	0.3	0.0	0.1	0.1	0.3	0.0	0.0	0.1
16	.09	.08(6)	0.2	0.1	0.0	0.1	0.0	0.4	0.0	0.1	0.1	0.3	0.0	0.0	0.1
17	.09	.08(7)	0.2	0.1	0.0	0.1	0.0	0.3	0.1	0.1	0.1	0.3	0.0	0.0	0.2
18	.09	.08(7)	0.2	0.2	0.1	0.1	0.0	0.1	0.0	0.2	0.1	0.3	0.0	0.1	0.2
19	.09	.08(7)	0.2	0.2	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.3	0.0	0.1	0.2
20	.09	.08(5)	0.1	0.2	0.1	0.1	0.1	0.4	0.0	0.2	0.1	0.3	0.0	0.1	0.1

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.09	.08(7)	0.2	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.3	0.1	0.2	0.2
02	.09	.08(7)	0.2	0.1	0.0	0.1	0.0	0.3	0.1	0.1	0.1	0.3	0.0	0.2	0.1
03	.09	.08(7)	0.2	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.1	0.3	0.0	0.0	0.1
04	.10	.08(8)	0.2	0.1	0.0	0.0	0.1	0.1	0.0	0.1	0.0	0.3	0.0	0.0	0.2
05	.09	.07(10)	0.2	0.0	0.0	0.0	0.1	0.1	0.0	0.1	0.2	0.3	0.0	0.1	0.1
06	.09	.08(7)	0.2	0.1	0.0	0.0	0.1	0.2	0.0	0.1	0.1	0.3	0.0	0.1	0.1
07	.09	.08(8)	0.3	0.1	0.0	0.0	0.1	0.2	0.0	0.1	0.3	0.3	0.0	0.0	0.1
08	.09	.08(8)	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.3	0.0	0.1	0.1
09	.09	.08(8)	0.2	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.3	0.0	0.1	0.2
10	.09	.08(9)	0.2	0.1	0.0	0.1	0.0	0.2	0.0	0.1	0.1	0.2	0.0	0.2	0.2
11	.10	.08(5)	0.2	0.1	0.0	0.1	0.0	0.4	0.0	0.1	0.1	0.2	0.0	0.2	0.2
12	.09	.08(7)	0.2	0.1	0.0	0.1	0.1	0.4	0.0	0.1	0.1	0.3	0.0	0.2	0.1
13	.10	.08(9)	0.2	0.1	0.0	0.1	0.0	0.4	0.0	0.1	0.2	0.3	0.0	0.1	0.2
14	.55	.38(25)	1.0	1.0	0.8	0.8	0.4	1.2	1.0	0.8	1.3	1.9	0.8	1.1	1.3
15	.08	.08(10)	0.2	0.1	0.0	0.0	0.1	0.2	0.0	0.1	0.0	0.3	0.0	0.0	0.1
16	.10	.08(11)	0.1	0.2	0.0	0.2	0.0	0.5	0.0	0.2	0.2	0.4	0.1	0.1	0.2
17	.09	.07(8)	0.3	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.3	0.3	0.0	0.0	0.1
18	.09	.08(7)	0.2	0.1	0.0	0.1	0.0	0.2	0.1	0.1	0.1	0.3	0.0	0.0	0.2
19	.10	.08(8)	0.1	0.2	0.0	0.1	0.0	0.5	0.1	0.2	0.1	0.4	0.0	0.2	0.3
20	.09	.08(7)	0.1	0.2	0.0	0.1	0.1	0.4	0.0	0.2	0.1	0.3	0.0	0.2	0.1

Appendix E (cont'd)

'Substrates' v. 1tpo (BACKBONE CONSTRAINED)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.39	.46	.42	.37	.55	.61	.58	.53	.16	.14	.15	.54	.50	.52	.46
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.62	.33(28)	1.9	0.6	0.6	0.2	2.6	3.2	1.2	1.4	2.6	1.8	1.2	0.7	1.2	1.5	0.8
02	.31	.08(28)	1.2	0.2	0.1	0.1	1.2	1.5	1.6	1.4	2.1	1.0	0.3	0.5	0.8	0.5	0.2
03	.73	.32(39)	2.0	1.0	0.5	0.2	3.2	3.4	2.2	1.5	2.1	1.8	1.3	1.6	1.6	1.5	0.9
04	.73	.36(36)	1.8	1.2	0.5	1.2	3.0	3.7	2.3	1.2	3.2	2.7	21	0.2	1.8	1.2	0.3
05	.86	.44(42)	2.4	1.5	0.7	1.3	3.9	4.4	3.0	2.0	3.5	2.8	2.2	0.3	2.0	1.3	0.5
06	.79	.54(22)	1.9	1.0	0.8	0.3	1.5	4.0	2.3	1.4	2.7	2.5	1.4	0.8	1.3	0.9	0.3
07	.80	.58(18)	1.5	1.1	0.8	0.1	3.6	3.8	1.9	1.5	2.6	2.4	1.2	1.1	1.4	1.1	0.5
08	.93	.56(29)	1.8	2.0	0.6	1.1	2.5	5.1	3.0	2.6	4.1	3.7	2.2	0.4	2.2	1.5	0.6
09	.94	.54(33)	2.4	1.6	0.7	1.0	4.1	5.1	3.6	2.0	3.5	2.9	2.0	0.5	2.2	1.4	0.6
10	.81	.50(24)	2.0	1.6	0.4	1.0	3.1	3.4	4.2	0.9	3.0	2.2	1.8	0.5	2.3	1.2	0.5
11	.76	.36(39)	2.2	1.3	0.5	0.7	3.0	4.0	1.8	1.1	3.4	2.5	1.9	0.8	1.2	1.2	0.9
12	.83	.52(28)	2.3	1.6	0.6	0.3	3.7	3.9	2.1	0.7	2.7	2.5	1.6	0.6	2.0	0.9	0.1
13	.70	.38(35)	2.0	0.8	0.5	0.4	3.0	3.2	1.1	1.1	2.7	2.4	1.7	0.9	1.3	1.1	0.4
14	.75	.43(27)	2.1	1.4	0.4	0.3	3.2	3.9	2.3	1.1	2.5	1.9	1.5	0.9	1.4	1.3	0.8
15	.74	.40(31)	2.0	1.1	0.6	0.3	2.5	3.4	1.7	1.5	3.0	2.9	1.8	0.8	1.4	1.4	1.0
16	.75	.38(30)	1.6	1.8	0.5	0.2	3.1	3.4	2.4	2.2	2.6	2.1	1.4	0.7	3.7	1.2	0.3
17	.86	.48(41)	2.3	1.4	0.6	0.5	3.4	4.7	2.6	2.2	2.5	2.3	1.5	1.1	1.8	1.4	0.4
18	.66	.31(38)	2.2	0.5	0.6	0.2	3.1	3.4	2.0	1.7	2.4	1.9	1.2	1.4	1.8	1.3	0.5
19	.58	.27(30)	1.6	0.9	0.5	0.2	2.3	3.3	2.2	1.6	2.3	1.7	0.9	0.6	1.6	1.4	0.6
20	.73	.42(26)	2.1	0.9	0.5	0.2	3.0	3.5	1.9	1.5	2.8	2.7	1.2	0.8	1.6	1.5	0.9

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.71	.43(25)	2.0	1.0	0.5	0.4	3.0	3.3	1.2	1.3	2.7	2.2	1.4	0.7	1.3	1.5	0.8
02	.87	.48(31)	3.2	2.2	0.4	0.5	2.9	2.8	2.7	1.0	2.7	2.4	1.6	1.5	3.3	1.5	1.1
03	.81	.39(40)	2.0	1.3	0.4	0.3	3.4	3.9	2.3	1.8	2.5	2.1	1.4	1.7	1.8	1.5	1.0
04	.88	.53(24)	1.7	1.8	0.3	1.6	2.2	4.7	4.9	1.3	3.3	2.8	2.1	0.1	1.8	1.0	0.2
05	.77	.48(21)	2.2	1.3	0.4	1.2	3.1	4.3	3.4	1.0	3.0	2.2	1.9	0.3	1.5	1.1	0.3
06	.80	.58(16)	1.9	1.0	0.7	0.8	3.5	4.0	2.3	1.4	2.6	2.4	1.4	0.8	1.3	0.8	0.3
07	.80	.61(14)	1.4	1.1	0.6	0.6	3.5	3.8	1.9	1.5	2.6	2.3	1.2	1.2	1.4	1.1	0.5
08	.74	.42(24)	1.8	1.6	0.4	0.8	3.3	4.1	2.7	1.2	3.3	2.8	2.0	0.4	1.9	1.3	0.5
09	.73	.44(22)	2.3	1.3	0.4	0.8	3.1	4.0	3.3	0.9	2.7	1.9	1.7	0.3	2.0	1.3	0.4
10	.66	.37(26)	1.9	1.1	0.4	0.3	2.7	3.3	1.3	1.1	2.7	1.9	1.4	0.8	1.3	1.4	0.8
11	.37	.11(32)	1.2	0.1	0.3	0.2	1.5	1.8	1.7	1.6	2.7	1.1	0.4	0.4	0.5	0.8	0.3
12	.84	.54(27)	2.3	1.5	0.6	0.7	3.7	3.9	2.2	0.9	2.8	2.6	1.6	0.6	2.0	0.9	0.1
13	.69	.37(35)	2.0	0.8	0.5	0.6	3.0	3.1	1.1	1.2	2.7	2.4	1.7	0.9	1.2	1.1	1.0
14	.66	.37(26)	1.9	1.1	0.4	0.3	2.7	3.3	1.3	1.3	2.7	1.9	1.4	0.8	1.3	1.4	0.8
15	.66	.35(33)	2.0	0.7	0.5	0.3	2.4	3.3	1.2	1.4	2.8	2.5	1.5	0.7	1.4	1.4	1.0
16	.81	.48(25)	2.1	1.0	0.4	0.6	3.2	4.2	2.2	1.7	3.1	3.1	1.4	0.9	1.6	1.5	1.0
17	.84	.50(31)	2.2	1.1	0.5	0.6	3.0	5.0	3.2	2.0	2.4	2.2	1.5	1.3	1.8	1.4	0.4
18	.68	.33(35)	2.2	0.6	0.5	0.3	3.2	3.4	2.1	1.9	2.6	1.9	1.2	1.5	1.9	1.3	0.5
19	.55	.22(33)	1.5	0.8	0.4	0.2	2.3	3.3	2.3	1.7	2.2	1.5	0.8	0.6	1.5	1.4	0.5
20	.81	.48(25)	2.1	1.0	0.4	0.6	3.2	4.2	2.2	2.2	1.7	3.1	1.4	0.9	1.6	1.5	1.0

Appendix F

'Substrates' v. 3ptp (BACKBONE CONSTRAINED)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.28	.30	.23	.25	.64	.64	.71	.64	.42	.42	.42	.34	.42	.32	.50
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.63	.25(27)	1.0	0.2	1.1	0.2	2.4	4.0	3.8	1.1	2.0	1.7	1.0	0.1	1.3	1.5	1.0
02	.77	.40(32)	1.8	0.6	1.1	0.2	2.6	5.3	3.3	1.7	2.6	2.4	1.7	0.4	1.4	1.5	1.3
03	.83	.44(29)	1.9	0.6	0.9	0.4	3.1	5.2	3.8	1.1	3.6	2.5	1.7	0.4	1.5	2.3	1.2
04	1.00	.55(25)	2.2	2.0	0.6	0.7	2.8	6.2	5.8	2.5	2.8	2.9	1.8	0.9	1.3	1.0	1.0
05	.67	.35(31)	1.6	1.2	0.5	0.2	2.9	3.4	2.9	2.1	2.2	1.9	1.6	1.1	1.0	1.5	0.6
06	.25	.07(18)	0.9	0.0	0.1	0.0	1.2	1.8	0.9	0.9	1.3	0.5	0.1	0.1	1.2	0.8	0.3
07	.58	.28(29)	1.3	0.8	0.4	0.2	2.1	2.8	1.6	1.4	2.0	2.2	0.9	0.8	1.4	1.0	1.1

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.84	.47(26)	1.5	0.8	1.0	0.5	2.7	5.5	4.0	1.3	2.5	2.4	1.3	0.4	1.4	1.7	1.3
02	.80	.43(30)	1.9	0.7	0.9	0.4	2.6	4.9	2.9	1.9	2.9	2.6	1.8	0.4	1.5	1.5	1.4
03	.89	.54(27)	2.0	0.8	0.7	0.8	2.4	5.8	2.9	1.7	2.9	2.5	1.9	0.6	1.5	2.1	1.2
04	.87	.55(22)	1.7	1.6	0.6	0.7	2.6	6.0	3.2	1.9	2.2	2.5	1.6	0.9	1.2	0.9	1.0
05	.69	.38(26)	1.5	1.2	0.5	0.4	2.7	4.4	2.7	2.2	2.1	2.0	1.4	1.0	1.5	1.7	0.5
06	.75	.37(31)	1.9	1.2	0.4	0.3	4.1	4.5	2.1	1.8	2.0	1.8	1.2	1.1	2.0	2.8	0.9
07	.70	.33(33)	1.5	1.0	0.5	0.1	3.1	3.0	4.1	2.0	1.8	1.6	0.9	0.8	1.5	0.9	1.2

'Substrates' v. 3ptp (N,C CONSTRAINED)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.28	.30	.23	.25	.64	.64	.71	.64	.42	.42	.42	.34	.42	.32	.50
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.84	.53(21)	1.4	0.7	0.1	0.4	2.3	5.9	3.8	1.3	2.5	2.0	1.2	0.6	1.4	1.6	1.2
02	.63	.27(33)	1.6	0.4	1.1	0.1	2.5	4.1	3.3	0.8	2.0	1.8	1.1	0.1	1.2	1.8	1.0
03	.82	.47(28)	1.9	0.7	0.9	0.4	2.5	5.4	2.6	1.5	3.0	2.5	1.9	0.5	1.4	2.0	1.1
04	.85	.52(23)	1.7	1.5	0.6	0.7	2.7	5.9	3.1	1.8	2.1	2.4	1.5	0.9	1.2	1.0	0.9
05	.68	.36(28)	1.5	1.2	0.5	0.2	2.7	4.4	2.7	2.2	2.1	2.0	1.5	1.0	1.5	1.7	0.5
06	.74	.36(34)	1.9	1.3	0.4	0.2	3.4	4.1	2.1	1.7	2.1	2.2	1.4	1.3	1.9	2.7	0.7
07	.70	.33(34)	1.5	1.1	0.4	0.3	3.1	3.0	4.1	2.1	1.8	1.6	0.9	0.8	1.5	0.9	1.3

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.76	.42(25)	1.2	0.5	1.1	0.5	2.5	5.5	3.9	1.2	2.2	1.8	1.1	0.4	1.3	1.6	1.1
02	.71	.40(31)	1.8	0.6	1.0	0.2	2.5	4.5	1.9	1.6	2.3	2.3	1.6	0.4	1.3	1.6	1.3
03	.89	.54(27)	2.0	0.8	0.7	0.8	2.4	5.8	2.9	1.7	2.9	2.5	1.9	0.6	1.5	2.1	1.2
04	.87	.55(22)	1.7	1.6	0.6	0.7	2.6	6.0	3.2	1.9	2.2	2.5	1.6	0.9	1.2	0.9	1.0
05	.69	.38(26)	1.5	1.2	0.5	0.4	2.7	4.4	2.7	2.2	2.1	2.0	1.4	1.0	1.5	1.7	0.5
06	.75	.37(31)	1.9	1.2	0.4	0.3	4.1	4.5	2.1	1.8	2.0	1.8	1.2	1.1	2.0	2.8	0.9
07	.70	.33(33)	1.5	1.0	0.5	0.1	3.1	3.0	4.1	2.0	1.8	1.6	0.9	0.8	1.5	0.9	1.2

Appendix F (cont'd)

'Enzymes' v. 2ptc (N,C CONSTRAINED)

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B			.52	.45	.45	.42	.45	.36	.50	.66	.49	.44	.45	.45	.50
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24
01	.09	.08(6)	0.0	0.1	0.0	0.1	0.1	0.2	0.0	0.1	0.3	0.4	0.0	0.1	0.1
02	.09	.08(6)	0.1	0.2	0.1	0.1	0.0	0.2	0.0	0.1	0.1	0.3	0.1	0.1	0.2
03	.09	.08(7)	0.4	0.1	0.0	0.1	0.1	0.2	0.0	0.1	0.1	0.3	0.0	0.1	0.1
04	.09	.07(9)	0.0	0.1	0.1	0.1	0.1	0.2	0.0	0.1	0.0	0.3	0.0	0.1	0.0
05	.09	.08(5)	0.2	0.1	0.1	0.0	0.0	0.1	0.0	0.1	0.1	0.3	0.0	0.0	0.1
06	.09	.08(4)	0.2	0.1	0.1	0.1	0.0	0.2	0.0	0.2	0.0	0.3	0.0	0.0	0.6
07	.11	.08(7)	0.1	0.1	0.0	0.4	0.3	0.2	0.0	0.2	0.0	0.3	0.1	0.1	0.6

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.09	.08(6)	0.0	0.1	0.0	0.0	0.1	0.2	0.0	0.1	0.3	0.3	0.0	0.1	0.1
02	.09	.08(7)	0.1	0.1	0.0	0.1	0.0	0.3	0.0	0.1	0.0	0.4	0.0	0.0	0.1
03	.09	.08(8)	0.3	0.1	0.0	0.1	0.1	0.1	0.0	0.1	0.3	0.3	0.0	0.1	0.1
04	.09	.07(9)	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.0	0.3	0.0	0.0	0.0
05	.09	.08(6)	0.3	0.1	0.1	0.0	0.0	0.1	0.0	0.1	0.1	0.3	0.0	0.0	0.1
06	.08	.08(0)	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.2	0.1	0.3	0.0	0.1	0.2
07	.11	.08(7)	0.1	0.1	0.0	0.4	0.3	0.2	0.0	0.2	0.0	0.3	0.1	0.1	0.6

'Substrates' v. 3ptp (N CONSTRAINED)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.28	.30	.23	.25	.64	.64	.71	.64	.42	.42	.42	.34	.42	.32	.50
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.70	.49(24)	1.4	0.9	1.1	0.4	1.8	1.6	3.0	2.0	1.9	1.3	0.9	0.6	1.5	1.8	1.2
02	.61	.32(32)	1.6	0.4	1.1	0.2	2.3	3.7	2.1	1.6	2.1	1.7	1.3	0.3	1.0	1.8	1.1
03	.69	.39(30)	1.7	0.5	1.1	0.3	2.6	3.9	2.4	1.9	2.4	2.2	1.6	0.3	1.2	1.9	1.0
04	.77	.47(24)	1.8	1.8	0.5	0.6	2.7	4.7	2.5	1.6	1.9	1.3	1.2	0.7	1.1	1.0	0.9
05	.67	.32(35)	1.6	1.3	0.5	0.2	2.5	3.5	2.9	2.0	2.2	1.8	1.5	0.8	1.5	1.8	0.6
06	.65	.34(30)	1.7	0.9	0.3	0.1	2.7	3.7	2.1	1.5	2.2	1.7	1.3	1.0	1.9	1.1	0.9
07	.61	.33(33)	1.5	0.9	0.4	0.2	1.9	2.0	3.2	1.3	1.2	1.2	0.7	0.8	1.3	0.9	1.1

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.49	.25(27)	0.8	0.2	1.2	0.2	1.6	1.6	2.7	1.5	2.0	1.0	0.7	0.1	1.3	1.5	0.9
02	.71	.45(22)	1.6	0.6	0.9	0.4	2.6	4.5	2.5	1.6	2.2	1.8	1.5	0.3	1.2	1.6	1.3
03	.77	.47(26)	1.8	0.5	0.9	0.7	2.6	4.2	2.8	1.5	3.0	2.6	1.0	0.3	1.3	2.0	1.0
04	.86	.57(21)	1.9	2.3	0.4	1.1	2.6	5.0	2.7	1.7	2.0	1.3	1.2	0.8	1.1	1.0	1.0
05	.67	.33(34)	1.6	1.3	0.5	0.3	2.5	3.5	3.0	2.0	2.2	1.8	1.5	0.8	1.5	1.8	0.6
06	.63	.34(30)	1.6	0.9	0.4	0.2	2.7	3.7	2.1	1.4	2.0	1.3	1.1	1.0	1.8	1.1	0.9
07	.61	.34(32)	1.5	0.9	0.4	0.1	1.9	2.1	3.2	1.4	1.2	1.2	0.7	0.8	1.3	0.9	1.0

Appendix F (cont'd)

'Enzymes' v. 2ptc (N CONSTRAINED)

			39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B			.52	.45	.45	.42	.45	.36	.50	.66	.49	.44	.45	.45	.50
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.17	.12(10)	0.1	0.2	0.3	0.2	0.2	0.4	0.0	0.0	0.1	0.2	0.0	1.2	0.2
02	.13	.12(06)	0.1	0.2	0.1	0.3	0.1	0.2	0.0	0.1	0.1	0.5	0.1	0.2	0.0
03	.13	.11(13)	0.3	0.2	0.1	0.2	0.1	0.3	0.0	0.1	0.0	0.5	0.2	0.1	0.1
04	.14	.12(05)	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.5	0.2	0.2	0.0
05	.13	.12(07)	0.3	0.2	0.2	0.3	0.1	0.1	0.1	0.3	0.1	0.4	0.1	0.0	0.1
06	.13	.12(05)	0.1	0.2	0.1	0.3	0.1	0.2	0.1	0.3	0.2	0.5	0.1	0.1	0.0
07	.18	.13(14)	0.2	0.2	0.0	0.3	0.2	0.1	0.1	0.4	0.2	1.0	0.5	0.6	0.6

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.16	.11(11)	0.1	0.2	0.2	0.1	0.2	0.6	0.1	0.1	0.4	0.3	0.1	0.6	0.0
02	.13	.11(10)	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.4	0.2	0.1	0.1
03	.14	.11(07)	0.5	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.4	0.1	0.1	0.1
04	.15	.12(04)	0.0	0.1	0.0	0.1	0.2	0.2	0.1	0.1	0.2	0.5	0.2	0.2	0.1
05	.13	.12(08)	0.2	0.2	0.2	0.3	0.1	0.1	0.1	0.3	0.1	0.4	0.1	0.0	0.1
06	.13	.11(07)	0.1	0.2	0.1	0.3	0.1	0.2	0.1	0.3	0.2	0.5	0.1	0.1	0.0
07	.18	.12(16)	0.2	0.2	0.0	0.3	0.2	0.1	0.1	0.4	0.2	1.0	0.5	0.6	0.7

'Substrates' v. 3ptp (NO CONSTRAINTS)

			17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.28	.30	.23	.25	.64	.64	.71	.64	.42	.42	.42	.34	.42	.32	.50
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.67	.52(18)	1.0	1.1	1.0	0.7	1.8	1.6	2.8	1.4	1.3	0.9	0.4	0.7	1.3	1.4	1.2
02	.40	.25(23)	0.9	0.7	0.8	0.1	1.3	1.7	1.1	0.8	1.9	1.0	0.6	0.1	1.3	1.3	0.5
03	.59	.49(13)	1.1	0.8	0.8	0.4	1.3	2.1	2.1	1.1	1.6	1.4	1.0	0.3	1.1	1.2	0.8
04	.61	.46(16)	1.2	1.4	0.8	0.7	1.4	2.6	1.9	0.3	1.7	0.6	0.6	0.7	1.0	0.8	0.7
05	.50	.33(25)	1.0	1.2	0.5	0.1	1.7	2.0	1.4	0.8	1.6	1.1	0.8	1.1	1.3	0.8	0.7
06	.48	.33(19)	1.1	1.1	0.3	0.2	1.4	2.1	0.7	0.7	1.5	0.7	0.6	0.8	2.0	0.9	0.9
07	.50	.33(21)	1.0	0.9	0.4	0.3	1.5	1.4	2.2	1.2	0.6	0.5	0.5	0.7	1.2	0.4	1.0

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.66	.54(12)	0.9	1.1	1.0	0.5	1.8	1.3	2.7	1.5	1.3	0.9	0.6	0.7	1.3	1.4	1.0
02	.59	.50(10)	1.0	0.8	0.7	0.3	1.4	2.2	1.7	1.1	1.7	1.3	0.9	0.2	0.9	1.4	1.0
03	.67	.60(08)	1.1	1.1	0.5	0.5	1.3	1.9	1.9	1.1	1.6	1.5	1.2	0.3	1.0	1.6	0.8
04	.71	.59(12)	1.2	1.8	0.5	0.2	1.5	1.9	1.4	0.9	1.5	1.0	0.8	0.8	1.5	1.4	0.6
05	.48	.30(26)	1.0	1.0	0.5	0.2	1.5	1.9	1.4	0.9	1.5	1.0	0.8	0.8	1.5	1.4	0.6
06	.45	.27(27)	1.1	0.9	0.3	0.1	1.3	2.1	0.7	0.7	1.5	0.7	0.6	0.8	1.9	0.9	0.7
07	.41	.22(27)	0.8	0.4	0.3	0.1	1.6	1.5	2.1	1.0	0.7	0.6	0.5	0.7	0.9	0.8	0.7

Appendix F (cont'd)

'Enzymes' v. 2ptc (NO CONSTRAINTS)

			39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B			.52	.45	.45	.42	.45	.36	.50	.66	.49	.44	.45	.45	.50
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.80	.68(10)	1.1	1.1	0.7	0.7	0.4	0.8	1.2	0.4	2.3	1.8	2.2	2.3	1.4
02	.42	.30(25)	1.0	1.0	0.6	1.2	0.9	1.0	0.7	0.4	1.1	1.5	0.7	1.0	0.9
03	.66	.56(14)	1.0	1.3	0.8	1.0	0.5	0.4	1.5	0.9	1.7	1.9	0.8	0.9	0.8
04	.64	.56(11)	1.0	1.1	0.9	1.1	0.8	1.0	1.3	0.5	1.4	1.8	0.8	1.0	0.8
05	.52	.40(19)	0.5	1.1	0.8	1.2	0.6	0.5	1.2	0.7	1.7	1.7	0.7	0.9	1.0
06	.51	.39(20)	0.6	0.9	0.8	1.2	0.5	0.8	1.1	1.1	1.5	1.7	0.6	0.8	1.0
07	.57	.42(19)	0.4	0.7	0.5	0.5	0.5	0.7	1.3	1.3	1.4	1.3	1.9	1.5	1.8

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.80	.63(17)	0.9	0.9	0.4	0.6	0.4	0.7	1.7	0.5	2.3	2.0	2.4	2.5	1.5
02	.67	.57(13)	1.4	1.4	1.0	1.1	0.6	0.5	1.3	0.6	1.4	1.6	0.8	0.9	0.8
03	.76	.68(08)	1.2	1.4	0.8	0.9	0.4	0.5	1.5	1.0	1.6	1.9	0.8	1.0	0.8
04	.75	.69(08)	1.2	1.0	0.9	1.0	0.7	1.2	1.4	0.8	1.5	1.6	1.2	1.1	0.9
05	.49	.37(21)	0.6	1.0	0.8	1.3	0.6	0.5	1.0	0.8	1.5	1.7	0.6	0.9	1.0
06	.46	.33(23)	0.5	0.8	0.7	1.2	0.4	0.8	1.0	1.0	1.5	1.6	0.5	0.8	1.1
07	.42	.25(28)	0.3	0.9	0.5	0.3	0.6	0.8	1.0	1.1	1.3	1.1	1.5	1.1	1.4

Appendix G

'Substrates' v. 1tpo (NO CONSTRAINTS)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.39	.46	.42	.37	.55	.61	.58	.53	.16	.14	.15	.54	.50	.52	.46
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.56	.48(9)	1.0	1.0	0.3	0.4	1.7	1.7	1.1	0.8	2.0	1.6	0.9	0.7	1.1	0.6	0.4
02	.59	.49(10)	0.9	1.2	0.5	0.4	2.3	2.4	0.9	0.6	2.0	1.6	0.8	0.8	0.8	0.6	0.6
03	.64	.54(10)	1.1	0.7	0.6	0.3	1.8	1.6	2.6	0.6	1.8	1.6	0.9	1.0	1.3	0.7	0.4
04	.65	.51(15)	1.4	0.7	0.6	0.3	2.0	2.2	0.2	0.4	1.8	1.6	1.1	0.9	1.5	0.8	0.3
05	.68	.52(16)	1.0	0.9	0.3	0.2	2.1	1.9	2.5	1.0	1.8	1.4	0.9	1.1	2.8	0.9	0.5
06	.49	.36(18)	1.0	1.6	0.4	0.6	1.6	1.0	0.7	0.6	2.2	1.3	1.2	0.6	1.3	0.5	0.3
07	.60	.52(9)	1.1	1.2	0.4	0.3	2.0	1.6	1.1	1.0	2.0	1.6	0.9	1.1	1.3	0.5	0.7
08	.46	.32(15)	1.0	0.3	0.4	0.3	1.7	2.1	1.1	0.5	1.8	1.1	0.8	0.6	1.6	0.7	0.2
09	.50	.33(22)	1.0	1.2	0.4	0.3	1.6	1.3	0.9	0.3	1.8	1.3	1.0	0.9	1.4	0.8	0.8
10	.60	.49(14)	0.9	1.8	0.5	0.3	2.2	1.8	1.2	0.6	1.6	1.4	0.7	1.2	1.4	0.6	0.4

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.54	.46(8)	1.0	1.0	0.3	0.7	1.6	1.9	0.7	0.3	1.9	1.5	0.8	0.7	1.1	0.6	0.4
02	.62	.52(11)	0.9	1.4	0.6	0.7	2.4	2.5	1.0	0.7	2.1	1.5	0.9	0.8	0.8	0.5	0.6
03	.63	.52(11)	1.1	0.7	0.5	0.6	1.8	1.6	2.6	0.6	1.8	1.6	1.0	0.9	1.5	0.7	0.4
04	.61	.47(14)	1.4	0.7	0.4	0.4	1.9	2.1	1.5	1.6	2.1	1.6	1.0	0.9	1.5	0.7	0.3
05	.62	.47(17)	1.1	0.8	0.4	0.5	2.3	1.9	1.0	0.8	1.8	1.4	0.9	1.1	2.3	0.9	0.4
06	.46	.33(18)	1.1	1.2	0.3	0.7	1.6	1.0	0.7	0.6	2.1	1.3	1.2	0.5	1.5	0.5	0.3
07	.70	.56(15)	1.1	1.5	0.6	0.6	2.4	3.0	1.6	0.8	1.9	1.7	1.0	1.0	1.3	0.6	0.6
08	.43	.28(16)	1.0	0.3	0.4	0.3	1.6	1.9	0.9	0.5	1.8	1.0	0.8	0.5	1.6	0.7	0.2
09	.51	.34(19)	1.1	1.7	0.3	0.5	1.6	1.4	0.6	0.6	2.0	1.3	1.0	1,0	1.3	0.8	0.8
10	.59	.48(16)	0.8	1.8	0.5	0.6	2.2	1.8	1.2	0.6	1.6	1.4	0.7	1.2	1.4	0.6	0.4

Appendix G (cont'd)

'Enzymes' v. 1tpo (NO CONSTRAINTS)

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B			.42	.42	.49	.44	.44	.37	.41	.44	.44	.39	.34	.42	.44
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24
01	.62	.50(18)	0.5	0.5	0.4	0.7	0.3	1.0	1.2	0.5	1.3	1.6	0.8	1.2	1.0
02	.61	.53(13)	0.7	0.6	0.3	0.5	0.2	0.7	1.1	0.4	1.2	1.3	0.8	1.0	0.9
03	.68	.58(14)	0.7	0.7	0.6	0.9	0.3	0.6	1.4	0.7	1.4	1.3	0.7	1.1	0.9
04	.69	.60(10)	0.6	0.6	0.4	0.6	0.5	0.8	1.1	0.5	1.5	1.7	1.0	1.1	1.1
05	.74	.59(17)	0.6	0.6	0.3	0.6	0.4	0.6	1.1	0.9	1.7	1.6	0.7	1.2	1.2
06	.57	.46(14)	0.5	0.5	0.5	0.8	0.3	1.0	1.1	0.6	1.1	1.7	0.8	0.8	0.9
07	.65	.54(14)	0.7	0.6	0.4	0.6	0.2	0.6	1.3	0.7	1.3	1.4	0.8	0.8	0.9
08	.48	.37(17)	0.3	0.5	0.3	0.7	0.2	1.0	1.0	0.5	1.0	1.3	0.7	1.2	1.2
09	.57	.39(24)	0.4	0.6	0.4	0.6	0.1	0.9	1.2	1.1	1.5	2.0	0.8	1.1	1.3
10	.67	.55(15)	0.6	0.6	0.5	0.6	0.2	0.3	1.3	0.6	1.6	1.8	0.8	1.2	1.0

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.60	.49(17)	0.4	0.5	0.4	0.6	0.2	1.1	1.2	0.5	1.2	1.6	0.9	1.2	1.0
02	.64	.56(10)	0.6	0.5	0.3	0.4	0.2	0.7	1.0	0.4	1.1	1.2	0.8	1.0	0.9
03	.66	.57(12)	0.5	0.6	0.5	0.8	0.3	0.5	1.3	0.6	1.3	1.3	0.7	1.1	1.0
04	.64	.51(17)	0.5	0.5	0.4	0.7	0.3	0.7	1.0	0.5	1.3	1.8	0.8	0.6	1.1
05	.69	.56(15)	0.5	0.6	0.5	0.7	0.4	0.6	1.0	0.7	1.6	1.9	0.8	1.1	1.2
06	.53	.39(21)	0.4	0.5	0.4	0.9	0.2	1.1	0.9	0.6	1.0	1.4	0.8	1.5	1.0
07	.72	.59(17)	0.8	0.6	0.3	0.5	0.4	0.5	1.5	0.7	1.6	1.1	0.8	1.0	0.9
08	.45	.33(20)	0.3	0.5	0.3	0.8	0.2	1.0	1.0	0.5	1.0	1.4	0.8	1.3	1.3
09	.56	.40(21)	0.3	0.6	0.4	0.6	0.2	0.8	1.2	1.1	1.5	1.8	0.8	1.5	1.1
10	.65	.50(23)	0.5	0.5	0.5	0.5	0.2	0.3	1.4	0.7	1.5	1.8	0.8	1.2	1.0

Appendix G (cont'd)

'Substrates' v. 1tpo (N CONSTRAINED)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.39	.46	.42	.37	.55	.61	.58	.53	.16	.14	.15	.54	.50	.52	.46
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.81	.52(25)	1.9	0.8	0.5	0.2	2.6	4.3	2.5	2.2	3.6	2.6	1.7	0.8	1.5	0.9	0.5
02	.78	.50(32)	1.8	0.9	0.5	0.3	2.5	3.1	2.8	2.1	2.8	2.4	1.4	1.5	2.2	0.7	0.6
03	.70	.48(19)	1.7	0.5	0.3	0.3	2.9	3.3	1.6	1.5	2.7	1.9	1.2	1.0	1.8	1.1	0.5
04	.84	.54(24)	2.3	0.8	0.3	0.2	3.7	4.0	2.0	1.8	3.1	2.6	1.7	1.1	1.9	0.9	0.5
05	.74	.45(25)	2.1	0.8	0.2	0.2	2.8	3.6	2.2	2.0	2.7	2.1	1.5	1.3	1.8	1.1	0.4
06	.87	.53(29)	2.0	2.5	0.3	1.1	3.1	3.6	2.7	1.6	2.9	2.3	1.9	0.8	1.7	0.7	0.3
07	.50	.25(28)	1.1	0.5	0.5	0.2	2.2	2.8	0.9	1.0	2.0	1.8	1.2	1.0	1.3	0.7	0.3
08	.82	.55(16)	2.1	0.6	0.3	0.1	2.5	4.3	2.3	2.0	3.6	2.4	1.7	0.8	1.7	0.9	0.3
09	.67	.35(27)	1.4	1.7	0.3	0.4	2.6	3.5	2.8	0.7	2.3	2.5	1.9	0.6	1.3	1.0	1.1
10	.87	.60(21)	2.1	1.0	0.6	0.3	3.2	3.6	2.2	2.0	3.5	2.7	1.6	1.3	1.9	1.0	0.7

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.78	.52(25)	1.7	0.8	0.5	0.8	3.0	3.8	1.8	1.7	3.0	2.5	1.5	0.9	1.5	1.0	0.5
02	.70	.46(25)	1.5	0.4	0.4	0.4	2.5	3.0	2.5	2.3	2.5	2.3	1.4	1.3	1.9	0.8	0.5
03	.72	.47(25)	1.8	0.5	0.3	0.6	3.0	3.3	1.7	1.5	2.7	2.0	1.2	1.1	2.0	1.1	0.4
04	.83	.48(31)	1.8	2.0	0.3	0.5	2.2	5.5	2.8	1.8	3.0	2.5	1.6	1.3	2.1	0.9	0.4
05	.79	.52(22)	2.1	0.8	0.3	0.5	3.4	3.7	2.3	2.2	2.9	2.4	1.4	1.3	1.8	1.1	0.5
06	.65	.34(27)	1.5	1.2	0.2	0.7	2.3	3.5	2.7	1.2	2.4	2.0	2.0	0.7	1.9	0.6	0.3
07	.65	.40(26)	1.4	0.7	0.3	0.4	2.7	2.7	2.2	2.1	2.4	2.3	1.5	1.3	1.3	0.9	0.6
08	.64	.36(21)	1.7	0.5	0.3	0.4	2.4	3.8	1.6	1.3	3.1	2.1	1.5	0.7	1.8	0.9	0.3
09	.68	.68(28)	1.4	1.7	0.2	0.6	2.6	3.5	2.0	1.2	2.4	2.5	1.9	0.6	1.4	1.0	1.1
10	.76	.51(21)	1.9	0.6	0.4	0.5	2.9	3.6	2.4	1.9	2.8	2.2	1.3	1.3	1.9	1.1	0.6

Appendix G (cont'd)

'Enzymes' v. 1tpo (N CONSTRAINED)

			39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B			.42	.42	.49	.44	.44	.37	.41	.44	.44	.39	.34	.42	.44
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.14	.11(11)	0.3	0.1	0.0	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.1	0.3	0.0
02	.14	.11(16)	0.3	0.1	0.3	0.3	0.2	0.1	0.2	0.3	0.2	0.5	0.8	0.1	0.1
03	.13	.11(11)	0.3	0.1	0.0	0.1	0.2	0.3	0.1	0.2	0.3	0.3	0.1	0.1	0.1
04	.12	.11(13)	0.3	0.1	0.1	0.0	0.1	0.1	0.1	0.2	0.1	0.3	0.2	0.1	0.1
05	.14	.11(10)	0.3	0.0	0.0	0.1	0.2	0.2	0.1	0.2	0.1	0.2	0.1	0.1	0.1
06	.13	.11(11)	0.3	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.4	0.2	0.1	0.2	0.2
07	.13	.10(13)	0.1	0.1	0.1	0.5	0.3	0.5	0.1	0.3	0.5	0.4	0.1	0.1	0.3
08	.13	.11(12)	0.3	0.1	0.0	0.2	0.2	0.2	0.0	0.2	0.1	0.2	0.1	0.3	0.0
09	.14	.11(7)	0.2	0.1	0.1	0.2	0.2	0.1	0.2	0.1	0.1	0.2	0.1	0.4	0.7
10	.14	.11(9)	0.3	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.1	0.0

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.12	.11(11)	0.2	0.0	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.3	0.2	0.1	0.1
02	.15	.11(13)	0.3	0.0	0.1	0.3	0.2	0.2	0.2	0.3	0.1	0.6	0.8	0.1	0.2
03	.12	.11(8)	0.3	0.0	0.0	0.2	0.2	0.3	0.2	0.2	0.1	0.3	0.1	0.1	0.1
04	.12	.11(8)	0.3	0.0	0.0	0.2	0.2	0.3	0.2	0.2	0.1	0.3	0.1	0.1	0.1
05	.14	.11(12)	0.3	0.0	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.3	0.1	0.1	0.0
06	.13	.11(8)	0.1	0.0	0.0	0.3	0.2	0.2	0.2	0.2	0.5	0.2	0.1	0.3	0.3
07	.14	.11(16)	0.2	0.0	0.1	0.4	0.2	0.3	0.1	0.3	0.4	0.5	0.1	0.3	0.3
08	.13	.11(12)	0.2	0.0	0.0	0.3	0.2	0.4	0.1	0.2	0.3	0.2	0.1	0.2	0.1
09	.13	.11(06)	0.2	0.0	0.1	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.1	0.2	0.7
10	.14	.11(09)	0.3	0.0	0.1	0.2	0.2	0.1	0.1	0.2	0.3	0.2	0.1	0.0	0.1

Appendix G (cont'd)

'Substrates' v. 1tpo (N,C CONSTRAINED)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.39	.46	.42	.37	.55	.61	.58	.53	.16	.14	.15	.54	.50	.52	.46
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.75	.49(18)	1.7	0.3	0.3	0.2	2.4	4.7	2.1	1.6	3.3	2.7	1.5	0.9	1.4	0.9	0.5
02	.81	.46(35)	1.9	0.8	0.4	0.4	3.1	3.7	2.2	2.3	3.1	2.7	1.5	1.2	2.0	1.0	0.5
03	.90	.59(23)	2.0	2.5	0.6	0.4	2.9	5.1	2.7	1.9	3.8	3.0	1.5	1.1	1.5	0.9	0.6
04	.74	.44(25)	1.7	0.6	0.2	0.1	3.3	4.4	1.8	1.4	3.0	2.6	1.7	1.2	1.6	0.9	0.4
05	.63	.34(25)	1.8	0.7	0.4	0.2	2.9	3.2	2.1	1.9	2.1	1.7	1.4	1.2	1.6	1.1	0.4
06	.76	.45(25)	1.7	2.2	0.3	0.7	3.0	3.6	2.7	1.7	2.7	2.3	2.4	0.7	1.6	0.5	0.3
07	.61	.36(26)	1.3	0.2	0.3	0.2	2.9	2.6	1.8	1.4	2.4	1.8	1.5	1.0	1.5	0.9	0.5
08	.84	.57(15)	1.7	0.9	0.3	0.1	2.5	5.5	4.0	1.0	2.9	2.1	1.4	0.8	1.7	0.9	0.4
09	.70	.35(29)	1.5	2.5	0.3	0.4	2.8	3.6	3.3	0.6	2.3	1.5	1.7	0.6	1.4	1.0	1.1
10	.73	.42(33)	1.8	1.9	0.3	0.2	3.1	3.6	1.7	1.7	2.8	2.6	1.4	1.3	1.6	1.1	0.7

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.64	.36(27)	1.5	0.3	0.4	0.4	2.3	4.2	1.6	1.2	2.7	2.1	1.4	0.9	1.5	0.9	0.3
02	.82	.55(21)	1.8	0.7	0.5	0.8	3.0	4.1	2.2	2.4	3.0	2.6	1.5	1.2	1.9	0.9	0.6
03	.89	.56(22)	2.1	2.5	0.5	0.7	3.6	5.0	2.7	1.8	3.6	3.2	1.5	1.0	1.4	0.9	0.5
04	.80	.57(12)	1.7	0.7	0.3	0.4	3.4	4.5	2.0	1.6	3.3	2.7	1.6	1.1	1.6	0.8	0.6
05	.78	.56(17)	1.9	0.7	0.5	0.4	3.2	3.2	2.5	2.7	2.6	1.9	1.3	1.2	1.7	1.0	0.6
06	.85	.60(18)	1.9	2.3	0.4	1.1	3.2	3.8	3.0	1.8	2.8	2.4	2.2	0.9	1.6	0.6	0.4
07	.75	.46(28)	1.6	0.4	0.4	0.6	3.3	4.1	2.6	1.3	2.7	2.0	1.5	1.2	1.7	0.9	0.7
08	.68	.41(18)	1.6	0.7	0.3	0.3	2.4	4.9	2.8	0.8	2.6	1.5	1.3	0.6	1.4	1.0	1.1
09	.69	.35(29)	1.5	2.5	0.2	0.5	2.8	3.6	3.3	0.6	2.3	1.5	1.7	0.6	1.4	1.0	1.1
10	.77	.53(22)	1.9	1.9	0.4	0.6	3.2	3.6	1.6	1.7	2.9	2.7	1.3	1.3	1.6	1.0	0.8

Appendix G (cont'd)

'Enzymes' v. 1tpo (N,C CONSTRAINED)

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B			.42	.42	.49	.44	.44	.37	.41	.44	.44	.39	.34	.42	.44
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24
01	.09	.08(8)	0.2	0.0	0.0	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.2
02	.09	.08(9)	0.2	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.3	0.1	0.1	0.3
03	.09	.08(9)	0.2	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.2	0.2	0.0	0.0	0.1
04	.09	.08(8)	0.2	0.0	0.0	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.2
05	.09	.08(7)	0.2	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.1	0.0	0.4
06	.10	.08(8)	0.2	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.3	0.2	0.1	0.2	0.6
07	.10	.08(8)	0.2	0.0	0.0	0.2	0.1	0.2	0.1	0.1	0.2	0.3	0.1	0.0	0.3
08	.09	.08(8)	0.2	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.2	0.0	0.1	0.2
09	.11	.08(7)	0.2	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.1	0.1	0.8
10	.09	.08(9)	0.3	0.0	0.0	0.1	0.1	0.2	0.0	0.1	0.2	0.3	0.1	0.1	0.2

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.10	.08(8)	0.2	0.0	0.0	0.2	0.1	0.3	0.1	0.1	0.2	0.2	0.1	0.1	0.3
02	.09	.08(10)	0.2	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.3
03	.10	.08(7)	0.3	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.2	0.5	0.0	0.1	0.2
04	.09	.08(6)	0.2	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.2
05	.09	.08(10)	0.3	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.0	0.3
06	.10	.08(6)	0.2	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.2	0.2	0.1	0.2	0.3
07	.10	.08(7)	0.2	0.0	0.0	0.1	0.1	0.2	0.1	0.1	0.2	0.3	0.1	0.0	0.2
08	.10	.08(8)	0.2	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.2	0.3	0.0	0.2	0.3
09	.11	.08(7)	0.2	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.1	0.1	0.8
10	.10	.08(8)	0.3	0.0	0.0	0.1	0.1	0.2	0.0	0.1	0.2	0.3	0.1	0.1	0.2

Appendix G (cont'd)

'Substrates' v. 1tpo (BACKBONE CONSTRAINED)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.39	.46	.42	.37	.55	.61	.58	.53	.16	.14	.15	.54	.50	.52	.46
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.97	.57(28)	2.0	0.8	0.7	0.6	3.6	5.8	4.2	2.0	3.6	3.6	1.8	0.9	1.6	0.9	0.8
02	.58	.28(29)	1.3	0.5	0.5	0.2	2.0	3.8	2.0	1.3	2.5	1.7	1.4	0.8	1.4	0.9	0.2
03	.69	.37(23)	1.8	0.6	0.3	0.2	3.3	4.5	2.8	0.8	2.4	1.8	1.3	1.1	1.3	1.0	0.3
04	.74	.43(23)	2.0	0.8	0.2	0.2	3.5	4.1	3.3	0.9	2.7	2.2	1.6	1.1	1.7	0.8	0.4
05	.89	.49(27)	2.4	2.1	0.3	0.1	3.4	5.0	3.2	2.5	3.1	2.5	1.6	0.9	1.8	1.2	0.7
06	.82	.44(31)	2.0	1.5	0.3	1.0	3.1	3.8	4.1	1.7	3.3	2.8	2.1	0.5	1.3	0.6	0.4
07	.99	.60(31)	2.1	0.8	0.8	0.8	3.8	4.5	3.2	2.4	3.7	3.9	2.1	1.6	2.3	0.8	1.2
08	.83	.49(20)	1.9	1.6	0.3	0.2	2.1	5.8	3.6	2.0	3.3	2.8	1.6	0.5	1.2	0.9	0.4
09	.76	.36(32)	1.5	1.1	0.3	0.4	2.8	3.8	2.5	2.0	3.6	3.5	2.1	0.8	1.5	1.0	1.3
10	.97	.57(28)	2.0	0.8	0.7	0.6	3.6	5.8	4.2	2.0	3.6	3.6	1.8	0.9	1.6	0.9	0.8

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.93	.57(22)	1.9	0.7	0.6	0.9	3.9	5.5	4.3	2.6	3.3	2.8	1.6	0.9	1.4	0.9	0.7
02	.87	.54(24)	1.4	0.4	0.5	0.9	2.9	5.0	3.4	2.6	3.7	3.3	1.8	1.1	1.7	1.1	0.7
03	.67	.37(22)	1.8	0.6	0.3	0.3	3.3	3.9	2.7	0.7	2.5	1.9	1.3	1.1	1.3	0.9	0.3
04	.96	.58(26)	2.1	1.3	0.7	0.8	3.5	5.3	3.6	1.9	3.7	3.6	1.9	1.1	2.0	0.9	0.6
05	.69	.32(22)	2.1	1.4	0.3	0.3	3.4	4.0	2.9	0.6	2.5	1.7	1.3	0.9	1.5	1.2	0.2
06	.74	.38(23)	1.6	2.2	0.2	0.8	2.7	4.5	3.3	2.9	2.9	2.0	2.1	0.6	1.4	0.6	0.3
07	.83	.53(25)	1.8	0.7	0.5	0.8	3.5	4.2	2.9	3.0	2.6	2.4	1.8	1.1	1.8	0.7	0.8
08	.87	.60(15)	2.0	1.6	0.6	0.7	2.5	5.6	3.8	1.0	2.8	1.9	1.3	0.6	1.3	0.8	0.4
09	.75	.36(32)	1.5	1.3	0.2	0.5	2.8	3.7	2.5	2.0	3.6	3.5	2.0	0.8	1.5	1.0	1.3
10	.82	.45(35)	1.6	1.3	0.4	0.5	3.2	5.6	3.5	2.4	2.6	2.2	1.3	1.3	1.7	1.1	0.7

Appendix H

'Substrates' v. 3ptp (NO CONSTRAINTS)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.28	.30	.23	.25	.64	.64	.71	.64	.42	.42	.42	.34	.42	.32	.50
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.56	.44(15)	0.9	0.9	0.9	0.3	1.4	2.1	2.1	1.2	1.7	1.3	0.8	0.6	0.9	0.8	0.4
02	.53	.38(20)	1.0	1.3	0.7	0.2	1.4	2.0	2.0	0.8	1.6	1.2	0.7	0.6	1.5	1.0	0.3
03	.67	.44(21)	1.5	1.4	0.4	0.4	1.7	1.6	2.3	0.9	1.1	1.1	0.7	0.6	3.5	1.2	0.5
04	.78	.55(20)	1.3	1.1	0.5	0.9	1.6	3.2	2.2	1.9	1.7	1.9	1.0	1.0	3.5	1.6	0.8
05	.58	.40(21)	1.0	1.3	0.3	0.3	1.2	2.8	1.7	1.5	1.5	1.7	0.8	0.2	1.8	1.5	0.6

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.52	.39(18)	1.0	0.8	0.8	0.3	1.2	2.0	2.0	0.8	1.7	1.4	0.8	0.6	0.9	1.0	0.3
02	.67	.52(15)	1.0	1.5	0.7	0.5	1.5	2.2	2.5	0.8	1.8	1.3	0.8	0.8	1.6	1.0	0.6
03	.54	.35(19)	1.1	0.8	0.3	0.2	1.7	1.6	1.9	0.6	1.1	1.1	0.6	0.5	2.9	1.6	0.6
04	.63	.39(25)	1.1	0.9	0.2	0.3	1.4	2.6	1.8	1.6	1.5	1.7	0.8	0.6	3.3	1.6	0.5
05	.51	.31(29)	0.9	1.0	0.3	0.3	1.0	2.4	1.5	1.2	1.4	1.5	0.9	0.1	1.8	1.7	0.5

'Enzymes' v. 1tpo (NO CONSTRAINTS)

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B			.42	.42	.49	.44	.44	.37	.41	.44	.44	.39	.34	.42	.44
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24
01	.67	.55(16)	1.2	1.3	1.1	1.2	0.4	0.6	1.0	0.4	1.5	1.7	0.9	1.5	1.0
02	.58	.50(12)	0.8	0.9	0.8	1.2	0.4	0.5	0.9	0.4	1.6	1.6	0.9	1.2	1.0
03	.73	.56(15)	0.6	0.8	1.1	0.8	0.1	0.4	1.1	0.8	2.6	1.7	1.9	2.2	2.4
04	.71	.60(13)	0.7	0.8	0.7	0.6	0.3	0.2	1.5	1.1	2.1	1.4	0.6	0.9	1.7
05	.58	.48(13)	0.5	0.7	0.6	0.9	0.3	0.4	1.2	0.6	1.8	1.6	0.7	1.0	1.7

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.60	.49(17)	1.0	1.0	1.0	1.1	0.4	0.6	1.0	0.4	1.4	1.7	0.8	1.3	1.0
02	.70	.65(05)	0.8	0.8	1.1	1.1	0.4	0.4	1.2	0.5	1.8	1.5	1.1	1.3	1.0
03	.60	.42(17)	0.5	0.5	0.7	0.7	0.2	0.5	1.0	0.6	2.2	1.5	1.6	1.9	2.1
04	.60	.53(08)	0.6	0.6	0.8	0.6	0.1	0.3	1.2	0.9	1.8	1.8	0.7	1.1	1.7
05	.51	.40(15)	0.3	0.6	0.5	0.9	0.3	0.4	1.1	0.4	1.6	1.8	0.7	1.0	1.7

Appendix H (cont'd)

'Substrates' v. 3ptp (N CONSTRAINED)

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.28	.30	.23	.25	.64	.64	.71	.64	.42	.42	.42	.34	.42	.32	.50
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15
01	.60	.30(31)	1.3	0.6	1.3	1.0	2.4	3.1	3.6	1.6	1.8	2.0	0.9	0.3	0.9	1.3	0.5
02	.86	.56(23)	2.2	1.9	1.0	0.5	1.7	1.7	4.4	2.9	2.6	1.8	0.4	0.8	1.8	1.2	0.8
03	.90	.46(31)	1.7	1.4	0.3	0.6	2.7	4.9	4.2	2.5	2.8	2.9	1.3	0.9	3.3	1.3	0.6
04	.74	.33(32)	1.5	0.9	0.3	0.2	2.7	3.7	3.1	2.5	2.7	2.8	1.3	0.5	2.9	1.6	0.6
05	.84	.47(33)	2.0	1.7	0.3	0.7	1.8	2.4	3.7	2.0	2.3	2.3	1.0	0.3	1.7	2.1	1.0

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.54	.28(23)	1.1	0.4	1.2	0.1	2.1	2.7	3.2	1.8	1.9	1.2	0.8	0.2	1.0	1.3	0.4
02	.86	.53(25)	1.8	1.9	0.9	0.6	2.8	3.7	3.3	2.4	2.8	2.7	1.4	0.8	1.6	1.3	0.7
03	.78	.36(29)	1.5	1.0	0.2	0.3	2.8	4.2	3.9	2.2	2.7	2.7	1.2	0.6	3.2	1.8	0.7
04	.94	.53(28)	1.9	1.4	0.5	0.4	3.3	4.5	3.2	3.0	3.3	3.2	1.6	1.1	2.9	1.3	0.6
05	.84	.47(32)	2.0	1.7	0.4	0.3	1.8	2.4	3.6	1.9	2.3	2.3	1.0	0.3	1.7	2.1	1.0

'Enzymes' v. 1tpo (N CONSTRAINED)

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
B			.42	.42	.49	.44	.44	.37	.41	.44	.44	.39	.34	.42	.44
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24
01	.14	.11(11)	0.2	0.1	0.2	0.5	0.2	0.1	0.1	0.1	0.1	0.3	0.1	0.7	0.4
02	.17	.11(14)	0.3	0.0	0.3	0.2	0.1	0.1	0.1	0.1	0.2	0.5	0.2	1.1	0.1
03	.14	.11(13)	0.2	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.6	0.3	0.1	0.4	0.7
04	.14	.11(13)	0.2	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.6	0.3	0.1	0.4	0.7
05	.16	.12(12)	0.2	0.2	0.1	0.3	0.0	0.1	0.1	0.2	0.2	0.5	0.3	0.3	0.8

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.14	.11(11)	0.2	0.1	0.1	0.6	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.6	0.4
02	.13	.10(14)	0.3	0.1	0.3	0.2	0.2	0.1	0.1	0.1	0.3	0.3	0.1	0.2	0.1
03	.14	.11(13)	0.2	0.2	0.1	0.0	0.1	0.1	0.1	0.0	0.2	0.4	0.1	0.4	0.7
04	.13	.11(14)	0.2	0.0	0.0	0.1	0.2	0.1	0.1	0.1	0.2	0.3	0.1	0.3	0.7
05	.16	.12(12)	0.3	0.2	0.1	0.3	0.0	0.1	0.1	0.2	0.2	0.5	0.3	0.3	0.8

Appendix H (cont'd)

'Substrates' v. 3ptp (N,C CONSTRAINED)

B			.28	.30	.23	.25	.64	.64	.71	.64	.42	.42	.42	.34	.42	.32	.50
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.99	.57(26)	1.9	1.0	1.6	0.5	2.9	5.5	4.9	2.4	3.4	3.2	2.0	0.6	1.1	0.8	0.8
02	.91	.51(29)	1.8	1.8	1.0	0.6	3.5	4.5	3.2	2.7	3.4	3.2	1.6	0.8	1.7	1.3	1.2
03	.72	.31(31)	1.6	0.8	0.4	0.1	3.0	3.2	3.7	2.2	2.7	2.1	0.8	0.4	3.0	1.7	0.5
04	.79	.34(32)	1.7	0.9	0.3	0.2	2.9	4.5	3.8	2.3	2.9	2.8	1.1	0.4	3.0	1.5	0.5
05	.91	.46(33)	1.8	1.7	0.3	0.4	3.0	4.4	4.3	2.5	3.2	3.1	1.3	0.3	1.6	2.0	0.5

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.92	.53(23)	1.8	0.9	1.6	0.7	2.6	5.5	4.0	2.6	3.7	3.5	1.8	0.7	1.0	0.8	0.6
02	.83	.46(27)	1.7	1.6	0.9	0.5	3.5	4.4	3.2	2.9	2.9	2.6	1.3	0.7	1.7	1.2	1.1
03	.72	.30(33)	1.6	0.8	0.3	0.2	3.1	3.2	3.7	2.2	2.7	2.1	0.9	0.4	3.0	2.0	0.5
04	.80	.35(32)	1.7	1.0	0.2	0.3	2.9	4.3	3.9	2.3	2.9	2.8	1.1	0.5	3.0	1.5	0.5
05	.83	.38(34)	1.5	1.3	0.2	0.3	3.0	4.4	4.0	2.2	2.9	2.8	1.2	0.2	1.8	2.1	0.5

'Enzymes' v. 1tpo (N CONSTRAINED)

B			.42	.42	.49	.44	.44	.37	.41	.44	.44	.39	.34	.42	.44
1tpo-2ptc			.35	.32	.16	.15	.21	.15	.24	.40	.20	.39	.36	.30	.24

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.09	.08(09)	0.3	0.0	0.1	0.0	0.1	0.2	0.0	0.1	0.2	0.2	0.1	0.1	0.3
02	.09	.08(08)	0.3	0.0	0.1	0.0	0.1	0.1	0.0	0.1	0.1	0.3	0.1	0.0	0.3
03	.12	.08(07)	0.3	0.1	0.1	0.4	0.1	0.1	0.0	0.0	0.2	0.2	0.1	0.2	0.7
04	.10	.08(06)	0.3	0.1	0.1	0.0	0.1	0.0	0.0	0.1	0.2	0.2	0.1	0.1	0.7
05	.10	.08(05)	0.4	0.1	0.1	0.0	0.1	0.0	0.0	0.1	0.3	0.3	0.1	0.1	0.7

CALCIUM IONS INCLUDED

sub	rms1	rms2	39Y	40H	41F	42C	43G	55A	143N	151Y	192Q	195S	196G	214S	215W
01	.09	.08(08)	0.3	0.0	0.1	0.0	0.1	0.0	0.0	0.1	0.2	0.2	0.1	0.1	0.1
02	.09	.08(08)	0.3	0.0	0.1	0.0	0.1	0.1	0.0	0.1	0.2	0.3	0.1	0.0	0.3
03	.12	.07(16)	0.2	0.1	0.1	0.4	0.2	0.1	0.0	0.0	0.2	0.2	0.1	0.1	0.7
04	.10	.08(06)	0.3	0.1	0.1	0.0	0.1	0.0	0.0	0.1	0.2	0.3	0.1	0.1	0.7
05	.10	.08(05)	0.3	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.3	0.3	0.1	0.1	0.6

Appendix H (cont'd)

'Substrates' v. 3ptp (BACKBONE CONSTRAINED)

			17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
B			.28	.30	.23	.25	.64	.64	.71	.64	.42	.42	.42	.34	.42	.32	.50
1tpo-3ptp			.12	.14	.15	.20	1.58	1.16	.52	.49	.25	.06	.17	.20	.24	.21	.15

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.53	.21(29)	0.9	0.3	1.0	0.1	1.9	3.3	3.6	1.8	2.3	1.6	0.7	0.1	1.2	1.1	0.5
02	.52	.23(34)	1.3	0.7	0.6	0.1	1.9	1.5	3.3	0.8	2.7	1.4	0.5	0.2	1.6	1.1	0.7
03	.34	.09(18)	0.4	0.1	0.1	0.0	1.3	2.0	2.5	1.2	1.8	0.7	0.1	0.0	1.8	1.1	0.3
04	.35	.10(21)	0.5	0.1	0.1	0.0	1.2	2.1	2.5	1.2	1.9	0.7	0.2	0.0	1.8	1.3	0.3
05	.46	.14(28)	0.7	0.2	0.2	0.1	1.6	1.6	3.8	1.8	2.2	1.6	0.3	0.2	1.9	1.4	0.4

CALCIUM IONS INCLUDED

sub	rms1	rms2	17V	19G	40H	74N	146S	147S	148G	149T	150S	151Y	152P	186E	188G	221Q	222K
01	.59	.29(25)	1.1	0.4	1.1	0.1	2.0	4.3	2.8	0.7	2.6	1.4	0.6	0.2	1.1	1.2	0.6
02	.49	.23(25)	1.1	0.5	0.6	0.0	1.7	1.5	3.3	0.8	2.7	1.3	0.4	0.2	1.7	1.1	0.6
03	.32	.09(17)	0.3	0.1	0.1	0.1	1.3	1.9	2.3	1.2	1.7	0.6	0.1	0.0	1.8	1.0	0.2
04	.36	.09(21)	0.5	0.1	0.1	0.1	1.2	2.1	2.6	1.2	2.2	0.9	0.3	0.0	1.8	1.3	0.3
05	.46	.14(28)	0.7	0.2	0.2	0.1	1.6	1.6	3.8	1.7	2.1	1.6	0.3	0.2	1.9	1.4	0.4

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