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A computational and experimental study of the inhibition of pyroglutamyl peptidase II by thyrotropin-releasing hormone analogs

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A Computational and Experimental Study of the Inhibition of Pyroglutamyl Peptidase II by Thyrotropin-Releasing Hormone Analogs

by

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Abstract

A Computational and Experimental Study of the Inhibition of Pyroglutamyl Peptidase II by Thyrotropin-Releasing Hormone Analogs

by

Richard G. Lanzara

Advisor: Professor Michael N. Liebman

Thyrotropin releasing hormone (TRH) is a tripeptide (pGlu-His-Pro-NH2) cleaved by the brain, synaptosomal, ectoenzyme pyroglutamyl peptidase II (PPII). PPII is a metallo-enzyme which likely contains a zinc atom at the active site. The interaction of TRH and nine TRH-analogs with PPII was examined by experimental and computational methods. The ten molecules were classified into three distinguishable experimental classes. These three classes were the: 1) substrates, 2) competitive inhibitors and 3) noncompetitive inhibitors. The experimental classes were modeled by comparing the electrostatic and steric patterns from the single molecules and their respective x-ray crystal environments. Four computational methods were used for the analyses of these three experimental classes. The four computational methods were the: 1) partitioned distance matrix method (PDM), 2) superpositioning by minimization of the root mean square distance between paired atoms (RMS method), 3) superpositioning by searching for the smallest absolute difference between the molecular electrostatic potentials (MEPs) within the crystal contacts (CCs) of the crystal environment (CC method) and 4) placement of the relative orientations within carboxypeptidase-A as a representative metallo-

protease. The CC superpositioning method superpositioned the calculated physicochemical properties of the molecules, rather than the atoms. From the comparisons of the competitive inhibitors with the substrates, all of the computer analyses were in general agreement. The PDM together with the RMS superpositioning methods identified constraints near the histidyl and prolineamide residues which were associated with the cleavage of the molecules by PPII. Regions surrounding the imadazole and prolineamide rings were found which may be important for the design of new inhibitors. Other regions near the proline ring may explain why the TRH analogs, RX74355 and RX77368 are poorly degraded *in vivo*. For the comparisons of the competitive inhibitors with the noncompetitive inhibitors, the four computer analyses allowed for differing interpretations. The CC superpositioning method produced alternative orientations for the noncompetitive inhibitors. These orientations may be germain to the further study of these inhibitors. This approach may also be extended to the studies of other classes of molecules.

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I dedicate this thesis to:

Daniel
Gregory
Joshua
Barbara
Gilda
Ruth
Dad
Mom
Chris
Sue
Jon
Rob
Bri
Nancy

I dedicate this thesis to the memory of Dr. Martin Sulkow, whose love, guidance and confidence in me propelled me to undertake this degree.

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List of Abbreviations

5-HT = 5 hydroxytryptamine (serotonin)

6-HT = 6 hydroxytryptamine

Analogs:

I = CG 3703 or (3R,6R)-6-methyl-5-oxothiomorpholin-3-ylcarbonyl-L-histidinyl-L-prolineamide

II = CG 3703 or (3R,6S)-6-methyl-5-oxothiomorpholin-3-ylcarbonyl-L-histidinyl-L-prolineamide

III = (4R)-2-oxothiazolidin-4-ylcarbonyl-D-histidinyl-L-prolineamide

V = 5-n-propylorotyl-L-histidinyl-L-prolineamide

VI = 5-n-bromoorotyl-L-histidinyl-L-prolineamide

VII or PheTRH = 5-oxo-L-propyl-L-phenylalanyl-L-prolineamide

DN 1417 = γ-butyrolactone-γ-carbonyl-L-histidinyl-L-proline amide

MK-771 = L-N-(2-oxopiperidine-6-carbonyl)-L-histidinyl-thiazolidine-4-carboxamide

PS-52 = 3-sulfhydral-5-hydroxy-1,2,4-triazine-L-histidinyl-L-proline amide

Ala = alanine

Asn = asparagine

CC = crystal contacts with the associated MEPs (determined from the crystal environment of the molecule)

CNDO = complete neglect of differential overlap

CPHNA = (N-1-carboxyl-2-phenylethyl(N^{im} benzyl)-histidyl-β-naphthylamide

EDTA = Ethelene diamine tetra-acetic acid

Gly = glycine

His = histidine

MEP = molecular electrostatic potential

pGlu = pyroglutamate (the cyclic form of glutamate)

PPI = Pyroglutamyl peptidase I

PPII = Pyroglutamyl peptidase II (EC 3.4.19-)

RMS = root mean square

TRH = Thyrotropin Releasing Hormone (also called TRF = Thyrotropin Releasing Factor)

Tyr = tyrosine

Val = valine

Introduction:

Thyrotropin releasing hormone (TRH) is a tripeptide (pGlu-His-Pro-NH2) which has several properties in common with neurotransmitters (Bauer, et al., 1981; Brownstein , et al., 1974; Hokfelt, et al., 1975; Kreider et al., 1981; Leppaluoto, et al., 1978; Taylor, et al., 1982; Warburg, et al., 1977; Winokur, et al., 1974). Pyroglutamyl peptidase II (PPII)(EC 3.4.19-), previously named "thyroliberinase" (Bauer, et al., 1981) or "pyroglutamate aminopeptidase II" (O'Connor and O'Cuinn, 1984, 1985 and 1986; Elmore, et al., 1990), is located in a particulate fraction of brain (Schock, 1977; Griffiths et al.; 1979, 1980, 1982; Hayes et al., 1979) and hydrolyses the pGlu-His peptide bond of TRH (Joseph-Bravo et al., 1979). PPII is the only synaptosomal membrane peptidase which specifically cleaves TRH (Elmore and O'Cuinn, 1987; O'Connor and O'Cuinn, 1985; Wilk, 1986; Wilk and Wilk, 1989). PPII has been characterized by its experimentally measured activities with other pyroglutamyl peptides and analogs of TRH (O'Connor and O'Cuinn, 1985; Elmore, et al., 1990; Lanzara, et al., 1989; Wilk and Wilk, 1989a and 1989b). Some of these structurally similar pyroglutamyl peptides and analogs of TRH display either competitive or noncompetitive inhibition of PPII (O'Connor and O'Cuinn, 1985; Lanzara, et al. 1989). A comparative analysis of these inhibitors with TRH would be desirable in order to describe the mechanism or mechanisms of inhibition and to design more potent inhibitors for PPII.

The x-ray crystal structure for PPII is currently unavailable. However without a three-dimensional structure, the active site of the

enzyme can be modeled by assuming that different substrates and competitive inhibitors display similar electrostatic and steric patterns which define the active site or pharmacophore (Marshall et al., 1979). The initial implementation of the pharmacophore concept is to describe, in a computational manner, the molecular interactions which are necessary for the binding of the molecule to the receptor site. The correspondence between the active regions in different molecules is correlated with the geometric arrangements of the regions which are common for the set of active compounds. This geometric arrangement serves as a potential receptor model which can be tested.

The beginning assumptions can be combinations of either a flexible or rigid site interacting with either a flexible or rigid ligand molecule. Because of the conformational freedom of some molecules, a multitude of three-dimensional conformations may be presented to the active site. Some of the problems introduced by assuming flexible molecules include an exponential increase in combinatorial analysis as well as an overwhelming amount of potentially useful information. Methods to reduce the information without losing relevant data are needed. As an approach to this problem Liebman extended the pharmacophore concept to the association of the physicochemical properties of the molecules within their crystal environment with the experimentally measured reactivities (Liebman, 1986). Liebman reduced the relative number of computations by encoding the information contained within the crystal site of the molecule and comparing similar regions for the set of active molecules. This method

is similar to the pharmacophore concept with the exception that the molecule's interactions with its surrounding crystal serve as the model for the binding site. For the active molecules, there should exist a set of common sites within the crystal. This set is composed of the interactions which the active molecules have in common within their respective crystal environments. These sets for all of the active molecules should have equivalent physicochemical properties for the essential binding sites.

The physicochemical properties of the molecules, rather than individual atoms, should be used as the template for a superpositioning of the molecules. The problem is to compare the physicochemical properties of the active molecules with those of the inactive molecules in order to discern the characteristics which are responsible for the active set. This comparison requires matching of the physicochemical properties and the active sites of the molecules with the experimentally determined groups or classes of molecules. The comparison involves a number of steps. The superpositioning of the molecules is a first, and nontrivial step, which by necessity involves assumptions about the relative orientations of the molecules. The root-mean-square deviation (RMS) is one of the most frequently used methods for comparing the structures of molecules. However, Liebman has cautioned about relying solely on the RMS method and has shown that other methods can be used to compare molecules (Liebman, 1982). Some of these other methods provide clearer and more logical comparisons of the molecules. In order to compare the physicochemical properties of the molecules, a

decision must be made about the type and magnitude of the cut-off criteria which are necessary for any comparison. Inclusion of cut-off criteria which are too strict may miss areas of important similarities among the molecules. Broader cut-off criteria risk the acceptance of areas which may contribute to the differences rather than to the similarities of the experimental observations made for these molecules.

During the analysis, the assumptions for the superpositioning may need modification, and therefore, a reorientation of the molecules should be done with a reassessment of the original assumptions. The importance of recognizing the need for this reassessment was demonstrated by Weinstein et al. in their analysis of 5-HT (5-hydroxytryptamine or serotonin) and 6-HT (6-hydroxytryptamine) at the serotonin receptor (Weinstein et al., 1985). By using the electrostatic orientation vector of the molecules as the criteria for the superpositioning, they demonstrated that an alternative orientation of the molecules could be found that provided a rational explanation for the different reactivities of the two molecules (Weinstein et al., 1985).

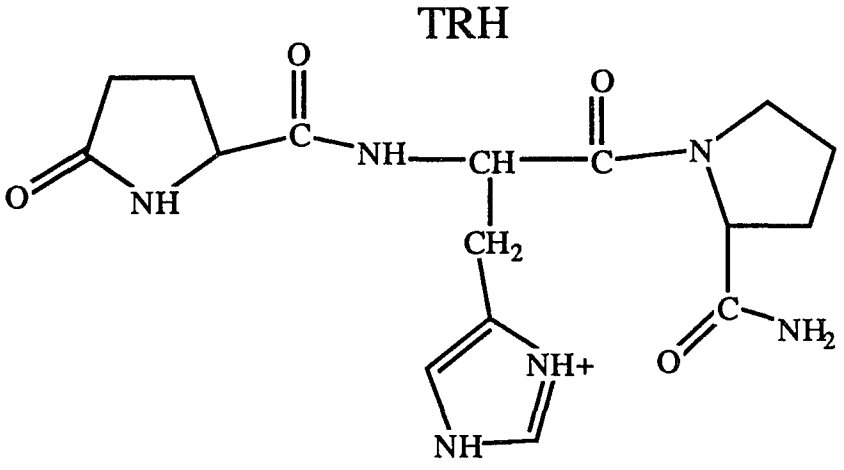
Since the interactions of TRH and its analogs with PPII have not been analyzed previously, the primary goals of this thesis are fourfold. The first goal is to test TRH and a series of TRH analogs, experimentally, with PPII in order to determine the type of inhibition, metabolism, rank order of potencies and apparent affinities of the molecules for PPII. The second goal is to construct computer models of TRH and the TRH-analogs with their respective crystal environments and to calculate the physicochemical properties of the molecules with and without their

respective crystal environments. The third goal is to encode within the computer models the patterned representations of the physicochemical properties of the molecules (molecular patterns) and compare these molecular patterns with the experimental results. This goal required the development of computer programs which were written in order to analyze the large amount of data. The fourth goal is to analyze the experimental classes for the calculated physicochemical similarities and differences. This last goal requires the comparisons of the identity elements within the molecular patterns for each of the experimental classes. Then the identity elements for one experimental class can be compared for similarities and differences with another experimental class. In this way, the elements which are different between the classes can be elucidated and may provide a reasonable understanding for the differing reactivities of the molecules. The first and second goals were direct applications of previous work. The third and fourth goals required the development of techniques which compared the molecules and their physicochemical properties by computational methods. These comparisons were successful in describing the differences among the various types of inhibitors which could account for many of the experimental results.

Background:

Thyrotropin-releasing hormone, TRH, also called thyroliberin, or thyrotropin releasing factor (TRF), was isolated from porcine hypothalamus by Schally's Group (Schally, 1966), and from ovine hypothalamus by Guillemin's Group (for a review see Guillemin, 1978 and Schally, 1978). Later, TRH was identified as the tripeptide, pGlu-His-Pro-NH2 (Bowers, 1970). Both Schally and Guillemin were awarded the 1977 Nobel Prize in physiology and medicine for their groundbreaking work in the isolation and characterization of TRH and other hypothalamic peptides (Guillemin, 1978 and Schally, 1978). The structure of TRH is shown in figure 1. TRH has both endocrine and

Figure 1: The structure of Thyrotropin Releasing Hormone (TRH)



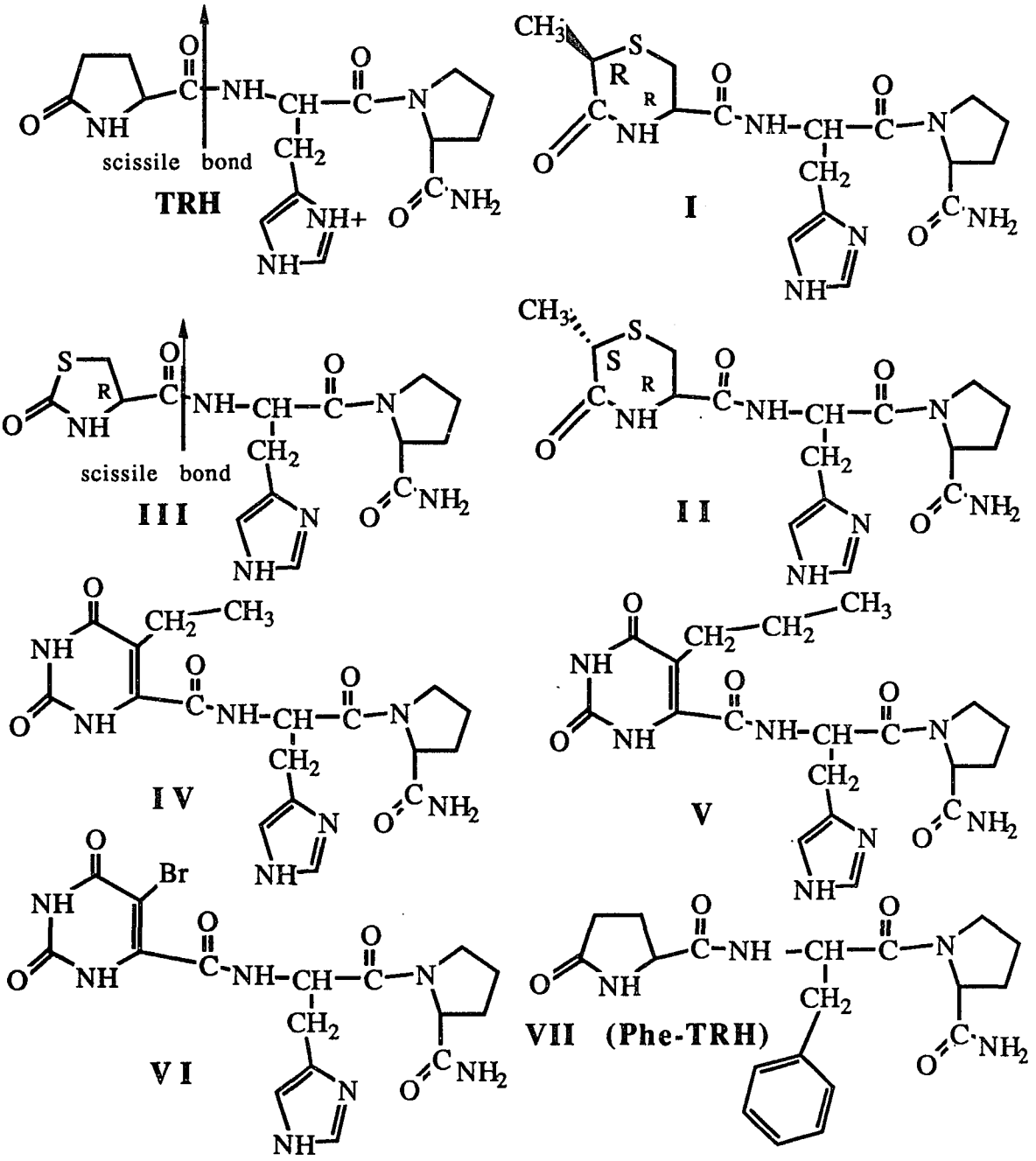
PYROGLUTAMYL HISTIDYL PROLINEAMIDE

neurotransmitter properties. It stimulates the release of thyrotropin and prolactin from the anterior pituitary and produces a range of excitatory effects in the central nervous system (Griffiths, 1983 and Jackson, 1982).

A neuroregulatory role for TRH was suggested by its receptor binding, synaptic localization, and behavioral effects (for reviews see Jackson, 1982, Griffiths, et al., 1983 and Metcalf and Jackson, 1989). In 1974, Winokur and Utiger showed that the hypothalamus contained only 31.2 percent of the total brain content of TRH (Winokur and Utiger, 1974). Brownstein et al. found TRH in regions of the rat brain outside of the hypothalamus and postulated that the peptide might function as a transmitter in the extrahypothalamic regions (Brownstein, et al., 1974). Using immunofluorescence, Hokfelt, et al. found TRH-containing nerve terminals in several motor nuclei in the anterior horn of the spinal cord (Hokfelt, et al., 1975). Kardon, Winokur and Utiger found a higher concentration of TRH in the anterior horn than in other areas of the gray or white matter (Kardon, et al., 1977). Cooper and Boyer in 1978 found that TRH produced an increase in evoked potentials and an increase in muscle tonus in the acute spinal cat (Cooper and Boyer, 1978). Bauer and Nowak found that direct application of TRH produced an excitatory effect on motor neurons in the frog spinal cord (Nicoll, 1977 & 1978; for a reveiw see White, 1989). The previous evidence, coupled with the finding that there are peptidases located within the brain that degrade TRH, strongly suggests that TRH is a neurotransmitter.

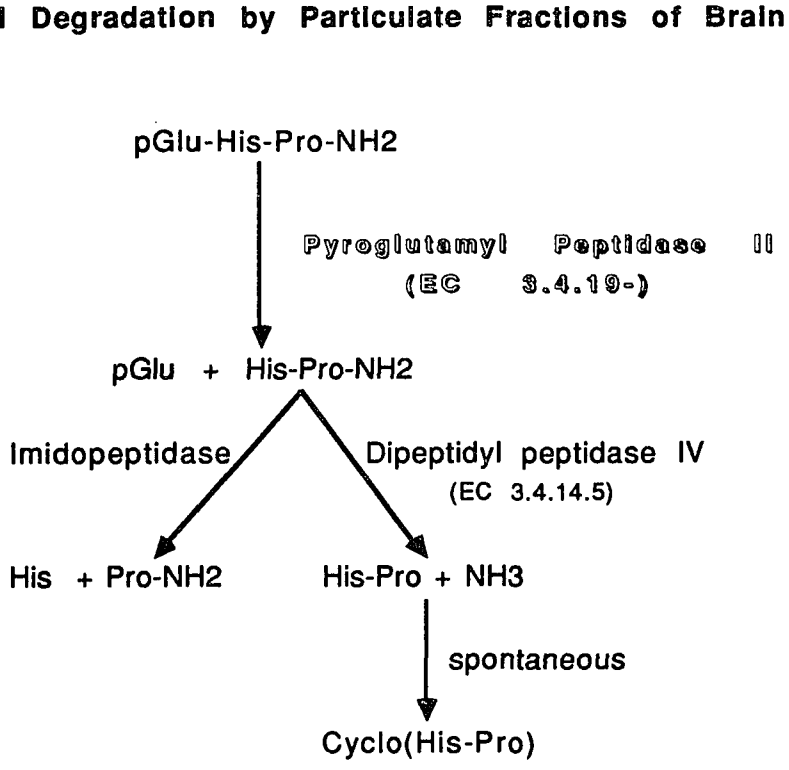
It wasn't until 1980 that Kamiya, et al. determined the x-ray crystal structure for TRH (Kamiya et al., 1980). Later in 1984, Eckle and Stezowski determined the x-ray crystal structures for several analogs of TRH (Eckle and Stezowski, 1985). Their purpose was to analyze the crystal structures of a series of central nervous system active TRH analogs (see Figure 2) in order to understand the spatial aspects of TRH receptor binding. Eckle and Stezowski used a least-squares superposition of the analogs, and found that the peptide backbones had a large degree of conformational similarity for the various analogs. Next, they examined the spatial distribution of potential donor (or acceptor) groups within the putative environment which could hydrogen bond with the appropriate chemical groups from the analogs. They termed the spatial distribution of these groups the hydrogen bonding environment. By constructing a composite hydrogen bonding environment, they detected regions where the bonding was common for the group of analogs. From their analysis of the analogs which had substitutions for the pyroglutamyl ring of TRH, they concluded that there were two regions, within the pGlu-substituted region, where receptor donors could hydrogen bond with the carbonyl oxygens of the analogs V, VI, VII (Phe-TRH) and TRH (Eckle and Stezowski, 1985).

Figure 2: The structures for TRH and seven TRH-analog molecules which have known x-ray crystal structures.



The enzymes which cleave TRH have been studied by many groups (for reviews see Griffiths, et al., 1989, O'Cuinn, et al., 1990, and Wilk, 1986 and 1989a). In the brain, the primary enzymes in the particulate fraction which cleave TRH are shown in Figure 3. A synaptosomal membrane bound dipeptidyl peptidase IV (EC 3.4.14.5) cleaves the postproline amide from His-Pro-NH2 to give His-Pro plus NH3 (O'Connor and O'Cuinn, 1986). This enzyme may compete with the formation of cyclic His-Pro, which is a byproduct of the reaction of PPII with TRH (Tadashi et al., 1978 and O'Connor and O'Cuinn, 1986).

Figure 3. Trh degradation in the brain particulate fraction.



Initially PPII was detected in the particulate fraction of the brain (Schock, 1977) and localized to the synaptosomal membranes (Greaney et al., 1980; Browne et al., 1981; Torres et al., 1986). PPII is a metalloprotease of molecular weight of 230 kilodaltons (O'Connor and O'Cuinn, 1984; Wilk and Wilk, 1989b). It is sensitive to inhibition by 8-hydroxyquinoline, 1,10-phenanthroline and EDTA which are metal chelating agents, but is insensitive to sulphydryl reagents (O'Connor and O'Cuinn, 1984; Wilk and Wilk, 1989b). Friedman and Wilk demonstrated that pyroglutamyl diazomethyl ketone, a nanomolar inhibitor of pyroglutamyl peptidase I (PPI), did not inhibit PPII (Friedman and Wilk, 1986). PPI, a soluble pyroglutamyl peptidase is a cysteine proteinase which is stimulated by dithiothreitol and EDTA, and has a broad specificity for pyroglutamyl peptides of varying size (for a review see Wilk, 1989). What distinguishes PPII from both PPI and the other TRH-degrading enzymes is its unique specificity for TRH and its primary localization within the brain as compared to its scarcity in other tissues (Elmore et al., 1990; Torres et al., 1986; Wilk, 1986).

Friedman and Wilk uncovered the presence of the more specific PPII by the inhibition of PPI and prolyl endopeptidase (Friedman and Wilk, 1986). *In vitro* or *in vivo* applications of the inhibitors of the soluble, TRH-degrading enzymes did not affect the levels of recoverable TRH (Charli et al., 1987). Recently, however, Charli et al. demonstrated a significant increase in the recovery of TRH released from brain slices after the application of CPHNA (N-1-carboxyl-2-phenylethyl(N^{im} benzyl)-histidyl-β-naphthylamide which is a reversible inhibitor of

PPII (Charli et al., 1989, Wilk, 1989). These studies on the substrate specificity of PPII coupled with other previously mentioned studies indicate that PPII may be the only known neuropeptide specific peptidase (Wilk, 1986 & 1989).

Studies with protein modifying agents indicate that the active site of PPII contains histidine, arginine and tyrosine residues (O'Connor and O'Cuinn, 1987). These residues are also found at or near the active site of other zinc containing proteases and have been implicated in the mechanism of cleavage for carboxypeptidase-A (Rees and Lipscomb, 1981, 1983; Liebman et al., 1985). The histidine residues are usually coordinated to the zinc atom while the tyrosine may play a role in catalysis (Rees and Lipscomb, 1981, 1983; Liebman et al., 1985).

The substrate specificity of PPII was studied by its interaction with a series of analogs of TRH, natural peptides (for reviews see Elmore et al., 1990 and Wilk and Wilk, 1989b) and with pyroglutamyl peptidyl naphthylamides (for a review see Wilk, 1989). Dipeptidyl naphthylamide pGlu-His-NA and the tetrapeptidyl naphthylamide pGlu-His-Pro-Ala-NA are not substrates (Wilk, 1989). This demonstrates that PPII peptidase activity is specific for peptide chain length. The placement of a benzyl group on the imidazole nitrogen of the histidyl residue does not affect binding to the enzyme or hydrolysis (Wilk, 1989). PPII cleaves the pGlu-His bond of TRH, but does not cleave the pGlu-His bond of LHRH, or the pGlu-His bond of neurotensin, eledoisin, bombesin, bradykinin-potentiating peptide 5a, B or 9a (O'Connor and O'Cuinn, 1985). However, LHRH(1-3) (pGlu-His-Trp)(O'Connor and

O'Cuinn, 1985) and pGlu-His-Trp-NA (Wilk, 1989) are substrates for PPII. Anorexogenic peptide (pGlu-His-Gly) and acid TRH (pGlu-His-Pro) are also substrates (O'Connor and O'Cuinn, 1985). PPII can cleave the pGlu-His-Pro-Gly and the 3'4'-dehydroPro derivative of TRH (Elmore et al., 1990), as well as the β-naphthylamide derivative of TRH (Wilk, 1989).

Elmore et al. also reported that cleavage occurs when either a -Trp or a -Pro-Gly group were substituted for the carboxy terminal prolineamide, but cleavage was blocked when -Trp-Ser-Tyr, -Gly, -Gly-NH2 or -Pro-Gly-NH2 were substituted for the carboxy terminal prolineamide. They also reported that the substrate specificity of PPII represents a significant departure from the rather broad specificity of other peptidases. Specificity studies indicate that PPII cleaves predominately only the pGlu-His-tripeptides (or small tetrapeptides such as pGlu-His-Pro-Gly), thus making the enzyme one of the most specific peptidases known.

The binding specificity of various compounds for PPII indicates that the larger peptides, such as LHRH, bind with affinities equal to or better than those of the substrates (Bauer et al., 1981; O'Connor and O'Cuinn, 1985). This implies that the larger inhibitors can have a greater number of favorable interactive energy sites with the enzyme than with the substrates or the smaller competitive inhibitors. The K_i of pGlu-His-NA is similar to the K_m of TRH-NA (pGlu-His-Pro-NH-β-naphthylamide)(Wilk, 1989a). An inhibitor may not need the prolineamide residue for binding to the enzyme. The compounds pGlu-

His-Trp and pGlu-His-Pro-Gly bind with K_i values of 170 and 125 μM respectively (Elmore et al., 1990) and are cleaved by PPII. This also suggests that the prolineamide is unnecessary for the cleavage of the molecule. However, the addition of the amide to pGlu-His-Pro-Gly to give pGlu-His-Pro-Gly-NH2 prevents cleavage of the compound and lowers the value of the K_i to 55 μM (Elmore et al., 1990). Also, the substitution of phenyl for the histidyl group prevents the cleavage of the molecule without appreciably altering the affinity (Lanzara et al., 1989, Elmore et al., 1990). Inhibitor studies demonstrate that PP II can bind either smaller or larger peptides with affinities similar to the affinity for TRH. Thus, selective substitution for the histidyl residue may prevent metabolism without changing the binding affinity. This may mean that the amide on the larger molecule can bind with some group or groups within PPII.

The detailed biochemical reactions that underly most biological processes are often unknown; however, the qualitative and quantitative effects of varying the components or parameters of the system are usually known. In order to model such processes, it is necessary to incorporate such qualitative effects into a model which is useful for interpreting the underlying processes involved. Inhibition experiments determine both the affinity an enzyme has for a particular molecule and whether the type of inhibition is competitive, uncompetitive or noncompetitive. By the classical description, a molecule which displays reversible, noncompetitive inhibition implies that there exists a second binding site on the enzyme. In the classical formulation,

noncompetitive inhibitors bind at a second site, however, for the compounds tested by O'Connor and O'Cuinn (O'Connor and O'Cuinn, 1985), pGlu-Val and pGlu-Ala displayed competitive inhibition whereas pGlu-His displayed a noncompetitive inhibition. O'Connor and O'Cuinn reported that all of the compounds which they tested were competitive inhibitors except for the pGlu-His. Recently, Elmore et al. (Elmore et al., 1990) reported that of the fifteen peptides which they examined all except pGlu-His were competitive inhibitors of PPII. However, pGlu-His was a noncompetitive inhibitor of PPII with a value for the K_i of 4.5 mM (O'Connor and O'Cuinn, 1985; Elmore et al., 1990).

By the classical model for noncompetitive inhibition, the K_i for pGlu-His is too large for a compound which binds to the active site in an irreversible manner. This implies the existence of a second site. However, the structural similarities of pGlu-His with the other competitive inhibitors and substrates suggests that there exists a paradox, because elements of recognition for a second, separate site appear on a molecule with elements which are identical with those for the substrates and competitive inhibitors. That these elements of recognition for a second site are selectively present in pGlu-His, but absent from pGlu-His-Pro, pGlu-His-Trp, pGlu-Val, pGlu-Ala, and pGlu-His-Gly (Elmore et al., 1990) is difficult to reconcile. Others have observed similar conundrums when applying the classical description of noncompetitive inhibition to binding at a site in common with competitive inhibitors (Leff & Martin, 1986, Auld and Holmquist, 1974, Rees and Lipscomb, 1981, Connolly & Trayer, 1979). Rees and Lipscomb

observed from the x-ray crystallographic structures that the binding of the indole ring of IAA (indole-3-acetic acid) occupies the S'_1 site which is the same site which is occupied by the tyrosine ring of Gly-Tyr (glycyl-L-tyrosine) in carboxypeptidase A (Rees and Lipscomb, 1981). Rees and Lipscomb observed only one binding site for IAA. Auld and Holmquist had previously reported that IAA inhibits the peptide hydrolysis of carboxypeptidase noncompetitively (Auld and Holmquist, 1974). Rees and Lipscomb mentioned the importance of their observation that both the noncompetitive inhibitor, IAA and the competitive inhibitor, Gly-Tyr, bind to the same region of the enzyme, but offered no kinetic model to describe the binding. Others have developed models which apply to the types of competition at the active site and for both reversible and irreversible inhibition of enzyme activity (Wang, 1990, Liu & Tsou, 1988).

In order to model how the spatial and physicochemical attributes of molecules can be compared within a series of structurally similar molecules several approaches have to be used. There are four levels of structural organization in proteins which can be distinquished as primary, secondary, tertiary and quaternary structure (Linderstrom-Lang, 1959). These terms refer to the amino acid sequence, the regular arrangements of the polypeptide backbone, the three-dimensional structure of the globular protein, and the structures of aggregates of globular proteins, respectively. Only relatively recently, has the analysis of these structural levels been quantitated and computed by such techniques as the linear distance plot, distance matrix, and

partitioned distance matrix (Liebman, 1980, 1982 & 1985). In a comparison of several molecules, Liebman found that the RMS prceedure was more susceptible to averaging and less informative about the modes of structural perturbation (Liebman, 1982).

The partitioned distance matrix (PDM) facilitates the comparison of a series of molecules by providing a unique representation of the distance pairs of elements within one partition to the elements of the corresponding partition for another molecule (Liebman, 1980, 1981, 1982 & 1985). The PDM provides a representation of the secondary and tertiary structure of these partitions within the molecules of interest. These and other spatial computations were used by Liebman et al. to study carboxypeptidase-A and were found to give useful information about the molecular structure of the molecule and the active site (Liebman et al., 1985). In this thesis, the PDMs were used in order to examine the spatial aspects of TRH and the TRH-analog molecules relative to the experimental classifications as substrates, competitive inhibitors or noncompetitive inhibitors. The association of the differences of the PDMs with the experimental classifications of the molecules is made for TRH and each of the TRH analogs with PPII.

An understanding of the reactive properties of the molecules requires the determination of the physicochemical properties, such as the electrostatic, van der Waals and dispersion energies, which underly specific interactions. In order to understand the reactive properties completely the nonspecific influences, such as the entropy and enthalpy of hydration, must be included into the calculation for the total free

energy of the system. Even with an x-ray crystal structure for PPII, this is currently impossible to calculate rigorously for such a large number of atoms. Although most of the free energy of biochemical systems may be determined by forces other than the electrostatic, the molecular electrostatic potential (MEP) is of fundamental importance in determining the orientation and the specificity of molecular interactions (Zauhar et al., 1988; Klapper et al., 1986; Matthew, 1985; Weinstein et al., 1985). The MEP is applicable to the studies of drug actions in a variety of systems and is an experimentally verifiable quantity (Weinstein et al., 1985). The importance of the molecular electrostatic potential (MEP) for molecular interactions has been mentioned by previous investigators (for reviews see Orttung, 1970; Tanford and Roxby, 1972; Matthew, 1985). MEPs can depict the chemical properties, and the molecular interactions of molecules of interest (Kollman and Hayes, 1976; Getzoff et al., 1983; Orttung, 1970; Tanford and Roxby, 1972; Matthew, 1985; McCammon and Karplus, 1980). The molecular properties that determine reactivity depend on the spatial distribution of intermolecular electrostatic forces. The electrostatic potential generated by the distribution of charges between atoms gives a description of the electrostatic force which one molecule can apply on another molecule. Those regions of space where the potential is negative will attract regions with positive charge distribution from another interacting molecule. The spatial orientation of two interacting molecules will orient in order to compliment the regions of positive

charge distribution and negative potentials or *vice versa* (Weinstein et al., 1985).

The calculation and display of the various molecular determinants such as MEPs, van der Waals' radii, solvent accessible surfaces, point charges, and other properties of interest are methodological problems which often require the display of a four-dimensional property (eg. the spatial coordinates and MEP) on a three dimensional display. The fourth parameter can be represented by color codes or displayed as lines of equipotential regions (Dean, 1987). A box and "reporter" plane technique have also been used to display the MEP (Lavery et al., 1982). These displays are more or less successful depending on whether the viewer can extract meaning from the data and interpret the results.

A problem with the displays of these properties is that the viewer is often overwhelmed with too much information that is irrelevant or confusing to analyze when comparing two or more molecules. Some form of data reduction must be performed in order to distill the critical facets of the data from the myriad amount of information which is available. This process of data reduction, by necessity, implies that some underlying assumptions must be made. For example, a spatial characterization might involve the location of some of the minima and maxima from the identification of common patterns which are recognizable from the underlying molecular properties. An analysis of these recognition patterns may provide a rationale for the realignment of the molecules and a logical explanation for similar reactivities from structurally different molecules (Weinstein, et al. 1985).

Another important problem for the analyses of molecular structures is the choice of superpositioning or alignment rules. Weinstein et al. made the observation that two sets of criteria used to superposition 5-HT (5-hydroxytryptamine or serotonin) and 6-HT (6-hydroxytryptamine) yielded different orientations of the molecules. They used as one criterion the alignment of the electrostatic vectors of the two molecules and concluded that the criteria for superpositioning should be based on the underlying molecular properties that determine the interaction, rather than criteria based solely on the molecular structure (Weinstein et al.,1985).

Liebman has previously demonstrated the feasibility of using the crystal environments and their physicochemical properties as a method for comparing and contrasting congeners of serotonin (Liebman, 1986). The spatial regions associated together with the physicochemical properties provide a template by which the comparisons of the molecules may be made. Patterns from these templates may be associated with the experimental reactivities for a particular class of molecules and used to determine a common physical basis for the observed reactivities. The association of these patterns with the experimental reactivities of the molecules is dependent on both choosing the physically meaningful orientations and calculating the physicochemical properties accurately for the molecules. With a more powerful computer and *ab initio* calculations the system could be more accurately characterized, however, the subsequent analyses across the experimental classes of these molecules would require an approach

which is similar to the approach used for this thesis. Therefore, the utility of this work remains in its ability to be useful for the analyses of more thoroughly characterized systems of molecules.

EXPERIMENTAL METHODS:

ENZYME ACTIVITY:

PPII was purified to apparent homogeneity from the particulate fraction of a rabbit brain homogenate (Wilk and Wilk, 1988). Enzymatic activity of PPII for the substrate pGlu-His-Pro-NA (TRH-naphthylamide) was monitored fluorometrically by measuring the release of 2-naphthylamine in an assay which coupled excess dipeptidyl peptidase IV with PPII (Friedman and Wilk, 1986). Under the conditions of the assays the velocity was linear with either enzyme concentration or time. Greater than 8% methanol inhibited the reaction, therefore, the methanol concentration was maintained at less than 6%. Briefly, the standard assay was carried out in a 0.05 M Tris-HCl buffer at pH 7.40. Varying amounts of substrate stock solution (10 mM) (1, 2 or 5µl) were mixed with 10 µl of DAPIV (0.105 µg/µl; Specific activity = 105 U/mg) in enough TRIS/HCl buffer to give 150 µl for each tube. To each tube was added extra buffer to bring the volume to 275 µl. The tubes were kept on ice and 25 µl of PPII enzyme preparation (952 U/ml; 0.04 mg/ml) was added to bring the final volume to 300 µl. The tubes were then incubated in a water bath at 37° C for 30 to 60 min. The amount of product formed was plotted verses the substrate concentration. The measured Km for the chromogenic substrate TRH-NA was obtained by a computerized analysis of the direct linear plot (Eisenthal R. and Cornish-Bowden A. 1974).

DETERMINATION of the DEGRADATION of TRH and the ANALOG MOLECULES by HPLC:

Degradation of the analogs was monitored by HPLC. The assay was performed with 20 to 40 µl of a 10 mM stock solution of the molecule to be tested with 50 to 100 µl of the PPII enzyme preparation (or buffer only for the control) with enough TRIS/HCl buffer to bring the final volume to 400 µl. The samples were incubated in a water bath at 37° C. Incubation mixtures contained the enzyme, PPII, a molecular analog of TRH and the Tris-HCl buffer. At various time intervals, a micropipette was used to withdrawal 10 µl samples and inject them into a C18 reverse phase column at varying time intervals. The solvent system for the column was an isocratic gradient of 10% methanol in a 0.05 M potassium phosphate buffer at pH 5.0. The elution tracings were calibrated with a known solution of cycloHis-Pro and TRH or the analog molecule.

INHIBITION STUDIES:

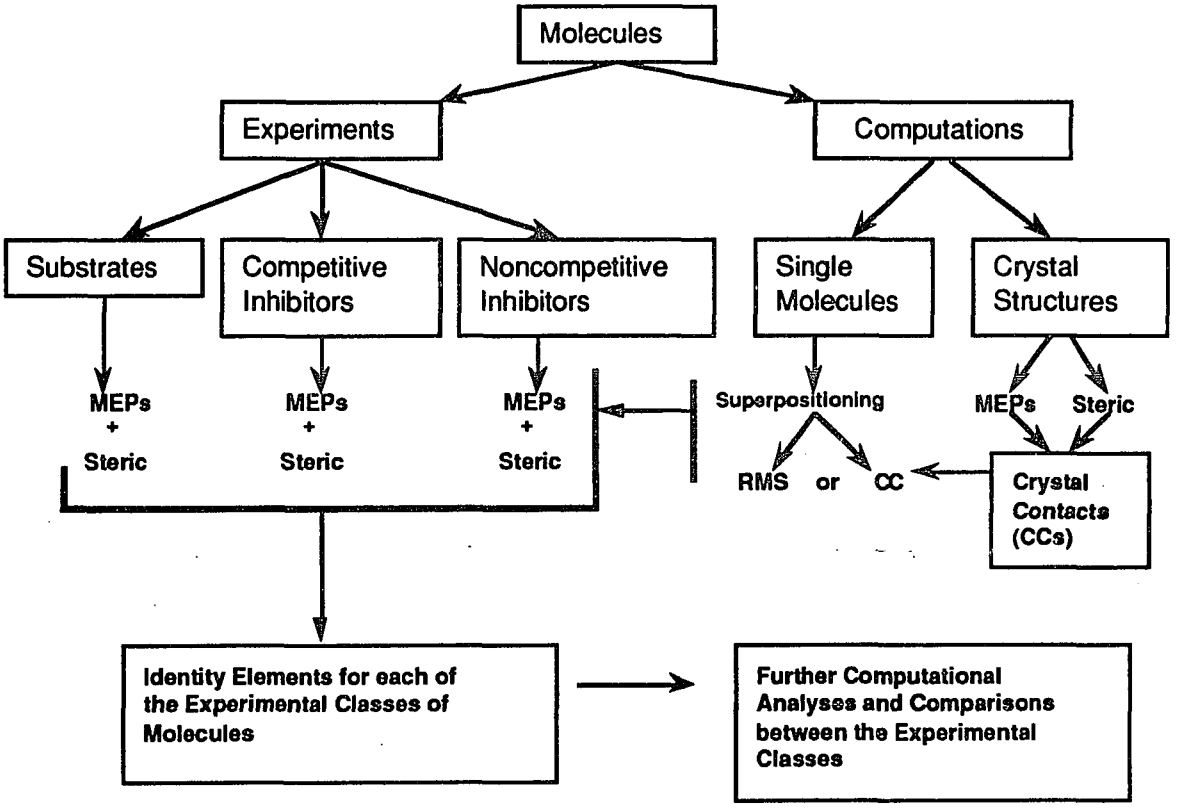
Briefly, the standard assay was carried out in a 0.05 M Tris-HCl buffer at pH 7.40. Varying amounts of substrate stock solution (10 mM) (1, 2 or 5µl) were mixed with 10 µl of DAPIV (0.105 µg/µl; Specific activity = 105 U/mg) in enough TRIS/HCl buffer to give 150 µl for each tube. The final substrate concentrations used were 33µM, 67µM or in some instances 167µM. To each tube was added varying amounts of the

inhibitors and extra buffer to bring the volume to 275 µl. The tubes were kept on ice and to each tube 25 µl of PPII enzyme preparation (952 U/ml; 0.04 mg/ml) was added to bring the final volume to 300 µl. The tubes were then incubated in a water bath at 37° C for 30 to 60 min. Competition of the molecules with TRH-NA was analyzed graphically by the methods of Dixon (Dixon, 1953) and Cornish-Bowden (Cornish-Bowden, 1974). From the comparison of the two graphs, the type of inhibition displayed by the molecules for the enzyme was determined unambiguously (Dixon et al., 1979).

COMPUTATIONAL METHODS:

The programs BANG, SYMTRN, ORTHO, LIMITS, DISDIF3, HOMLAL, FORDUPNEW, INDOTEST, FROMVWA, SUMCHG, READCG and VDWCEL3 were kindly supplied by Dr. M. Liebman. The programs, SUMMABAT3, A, A1, B, B1, T1, PN, Q, S1, S2, N1, C1, D1, D2, PN1, EPAD, ROTAMTXB, QMCTORUG, ROTAS1 and S3 were written for this thesis. These programs were used to build the crystal environments of the molecules and to calculate the molecular electrostatic potentials, point charges and steric volumes as well as analyze the experimentally determined classes for similarities and differences. The identity elements of the molecular patterns for each of the experimental classes were compared to the identity elements from one experimental class with the identity elements from a different experimental class. The overall approach is outlined in the diagram on the next page.

Interface of the Experimental Data with the Computations

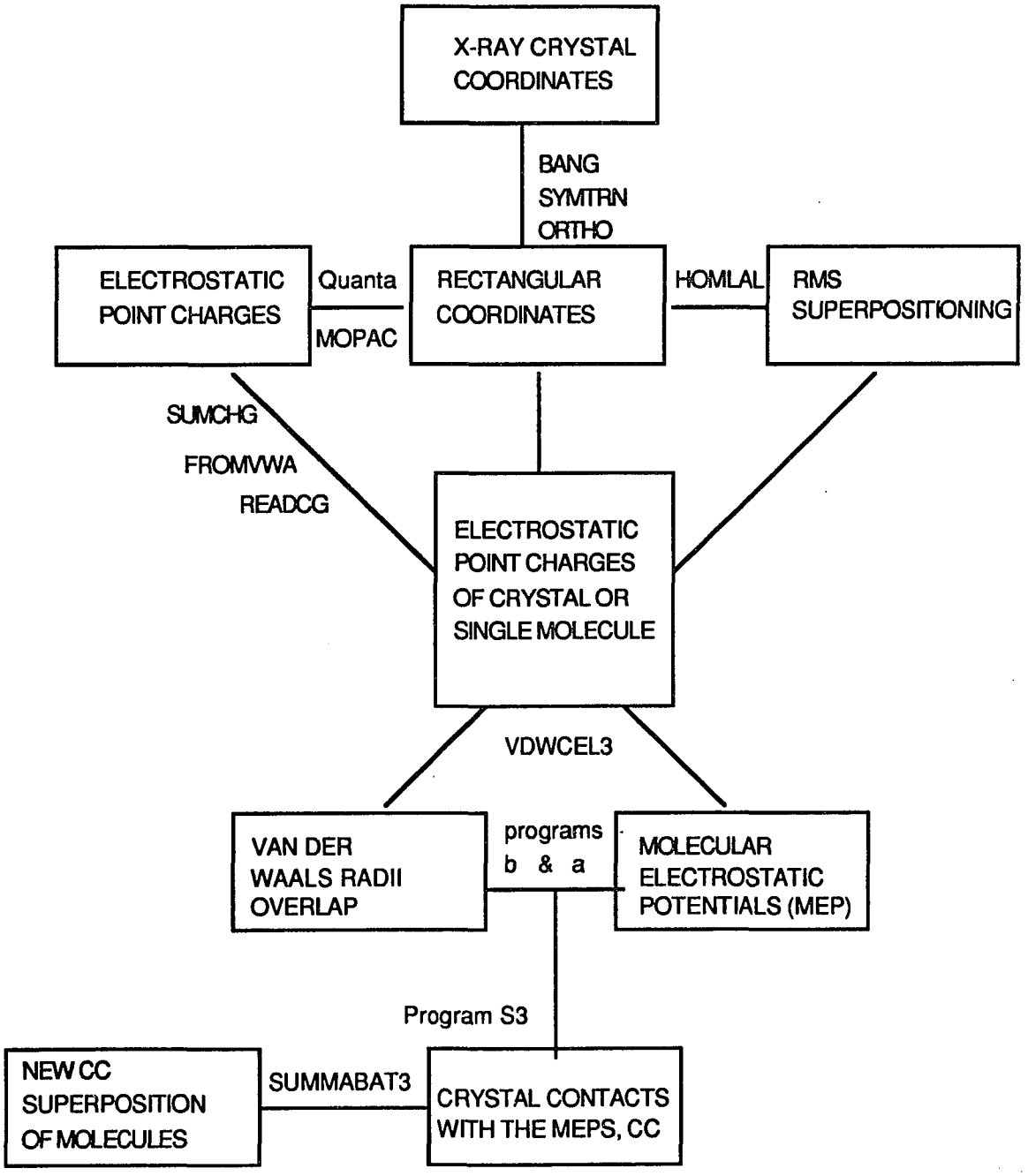


GENERATION OF THE MOLECULES AND THEIR CRYSTAL STRUCTURES:

The fractional atomic coordinates of the TRH-analogs are taken from Eckle and Stezowski (Eckle and Stezowski, 1985). The fractional atomic coordinates for TRH are taken from Kamiya, et.al. (Kamiya et al., 1980). The resolution of the molecules ranged from 0.869 to 0.619 Å. The R values of the structures ranged from 0.079 to 0.044.

The crystal structures are generated first by transformation of the fractional coordinates into orthogonal coordinates. The programs BANG, SYMTRN, and ORTHO are used to build either the single molecules or the crystal environments for each of the molecules. The program, BANG, generates the symmetry table for a central molecule within the surrounding crystal environment. The symmetry elements which are output from BANG are entered into the program SYMTRN which generates the crystal coordinates within a fractional coordinate system. The program ORTHO transforms these fractional coordinates into orthogonalized coordinates which can be displayed on a graphics terminal (see Figure 4). A routine visual check of the molecules and crystal structures is performed on one of the graphics display terminals (Megatek 9200 or SILICON GRAPHICS) running one of the graphics display programs MERLIN or POLYGEN'S QUANTA. QUANTA© is software which was developed by the Polygen Corporation (Waltham, Massachusetts).

Figure 4: Flow chart of the programs which were used to construct the molecular crystal structures, electrostatic charges and superpostion the molecules.



PARTITIONED DISTANCE MATRIX ANALYSES:

Partitions of the molecules were created for the nonhydrogen atoms as described previously (Liebman, 1980, 1981 and 1982). Briefly, the twenty-six, nonhydrogen atoms (13 pairs) from either TRH or analog III were paired for the PDM analyses with the analogous atoms choosen from the analog molecules. Atoms in the analogs which could not be matched with a corresponding atom within TRH or analog III were eliminated. A thirteen by thirteen matrix was generated for each of the comparisons. All of the comparisons for the analog molecules were compared to analog III which is a substrate for PPII. For each experimental class of molecules, the regions of absolute differences were examined which could differentiate between the experimental classes. After determining these regions of differences within the matrices, the corresponding atoms which contributed to these differences were outlined on a stick diagram of the TRH molecule.

The program DISDIF3 determines the distances within selected partitions for two molecules which are to be compared. The distances are determined from each atom to the other atoms within the partition and then subtracted from the similar distances from the corresponding partition of the second molecule. The program DISDIF3 tabulates these partitioned distances into both the average signed (avesig) and averaged absolute (aveabs) matrices. Regions are identified which associate with either the competitive inhibitors (both cleaved and not cleaved) and the noncompetitive inhibitors of PPII.

STRUCTURAL SUPERPOSITIONING OF THE MOLECULES:

The RMS superpositions of the molecules by minimizing the root mean square distances between the selected atomic positions was done by the program HOMLAL. HOMLAL performs a rigid molecule reorientation of one molecule onto another (Liebman et al., 1985). The two sets of atomic positions encompassing the scissile peptide bond and the neighboring alpha carbon atoms for each of the molecules are superimposed by a series of transformations. The transformations are performed by a series of rotations and the best superposition is found which is the minimum in the root mean square deviation (rms) between the sets of atomic positions. HOMLAL superpositions the selected atoms which included the scissile peptide bond and the neighboring carbon atoms for each of the molecules. TRH and all of the analog molecules were superpositioned initially by this method.

In order to superimpose the two sets of crystal contacts for two different molecules, the program SUMMABAT3 was written. The program reorients one set of crystal contacts relative to another set and determines the smallest absolute difference between the two sets of crystal contacts. The smallest absolute difference is determined by examining for the nearest point within the grid size of 0.5 Å. SUMMABAT3 stores the rotation and translation matrix of the positional change from the original position into a reference file. Only those positions which are succeedingly improved (those with the smaller

absolute differences) are stored in the reference file. The reference file is later examined and these positions are regenerated from the original position of the molecule by entering the positional matrix parameters into the program ROTAMTXB.

All of the crystal contacts are determined by combining the MEP and steric output from the program VDWCEL3. The regions of van der Waal's contacts which the central molecule makes within the crystal environment are combined with the MEPs from these regions. This is what is subsequently refered to as the crystal contacts (CCs).

CALCULATION OF ELECTROSTATIC POINT CHARGES FOR EACH OF THE ATOMS IN EACH OF THE MOLECULES:

POLYGEN'S QUANTA CNDO program is used to calculate the CNDO (*complete neglect of differential overlap*) atomic charges. The internal coordinate files are generated on the SILICON GRAPHICS computer running POLYGEN'S QUANTA program version 1.1. All the calculations are checked by running the programs FROMVWA and SUMCHG to determine the net charge for each of the molecules. The program READCG is then run in order to read the charges in the FROMVWA format from the CNDO calculations onto the orthogonalized coordinate files (see figure 4). By these methods the charges for the molecules alone and the entire crystal are constructed. Although there exists some controversy about the accuracy of CNDO charges, the major regions for the molecules which were studied for this thesis were the large

electronegative regions which surrounded the carbonyl oxygen atoms. MNDO (*modified neglect of differential overlap*) charges were also calculated for these molecules and some differences between the CNDO and MNDO charges were found, however, the relatively large electronegative regions around the oxygens remained relatively constant between the MNDO or CNDO calculations. Therefore, for consistency, the CNDO charges are used throughout for the calculation of the MEPs and for the subsequent comparisons of the molecules.

PLACEMENT OF THE HYDROGENS WHICH WERE NOT ASSIGNED TO WATER OXYGENS WITHIN THE CRYSTAL STRUCTURES:

The placement of the hydrogens, which are missing from the water oxygens because of disorder in the x-ray crystal structure, was accomplished by calculating the nearest neighbors within three angstroms in order to find the most likely atoms to form hydrogen bonds. The hydrogens are then generated so that the H-O-H angles are within the normal ranges for the water, oxygen molecules with hydrogen atoms within the crystals. After placement, the orthogonalized coordinates are transformed to fractional coordinates using a modification of the program HOMLAL. The program BANG then finds any new symmetries. If necessary, the structures are generated again as explained above (see Generation of the Molecules and Their Crystal Structures).

DETERMINATION OF THE CRYSTAL ENVIRONMENTS:

The orthogonalized coordinate files are analyzed by the program LIMITS to find the center and the largest dimensions of the molecule. The consensus interval for each of the molecules is calculated to include the x, y, and z directions plus six Angstroms added to each direction which defines the "box" of the local crystal environment (Liebman, 1986). The program Bang generates the symmetry tables at 4, 5, 6 and 7 Å around the central molecule within the crystal environment. Then the crystal symmetry is used to construct the surrounding local environment of each molecule by sampling a 0.5 x 0.5 x 0.5 Angstrom sized grid over the entire consensus interval surrounding the central molecule. The grid size determined the resolution limit for the analyses and was a compromise between the computer time and space with the resolution of details.

ASSIGNMENT OF STERIC PROPERTIES AND CALCULATION OF THE MOLECULAR ELECTROSTATIC POTENTIALS:

The molecular electrostatic potentials (MEPs) are calculated by using the partial atomic charges from the CNDO calculations with the program VDWCEL3 in order to calculate the MEPs for each of the molecules. The MEPs are generated for the separate molecules by sampling the effective charge experienced by a point positive charge as it moves through the regions of the molecule within a cubic grid. The

output is a series of two-dimensional grids with the quantity and sign of the MEP given for each point on the grid. These MEPs from the program VDWCEL3 were compared to those MEPs calculated by Polygen's QUANTA program and were found to be in general agreement. When combined, these two-dimensional grids form a three-dimensional representation of the MEPs.

The steric properties of the molecules were calculated from the program VDWCEL3 by the assignment of the van der Waals surface of each element in the molecule to a region of a cubic grid as to its occupancy, vacancy, or overlap with the other labeled elements of the crystal. The data reduction, display and analyses of the molecules with the associated physicochemical properties were accomplished with the programs (A, B, A1, B1, C, D, PN and Q) which interface the VDWCEL3 output with the Silicon Graphics Workstation running Polygen's Quanta Program. These programs are described in more detail below.

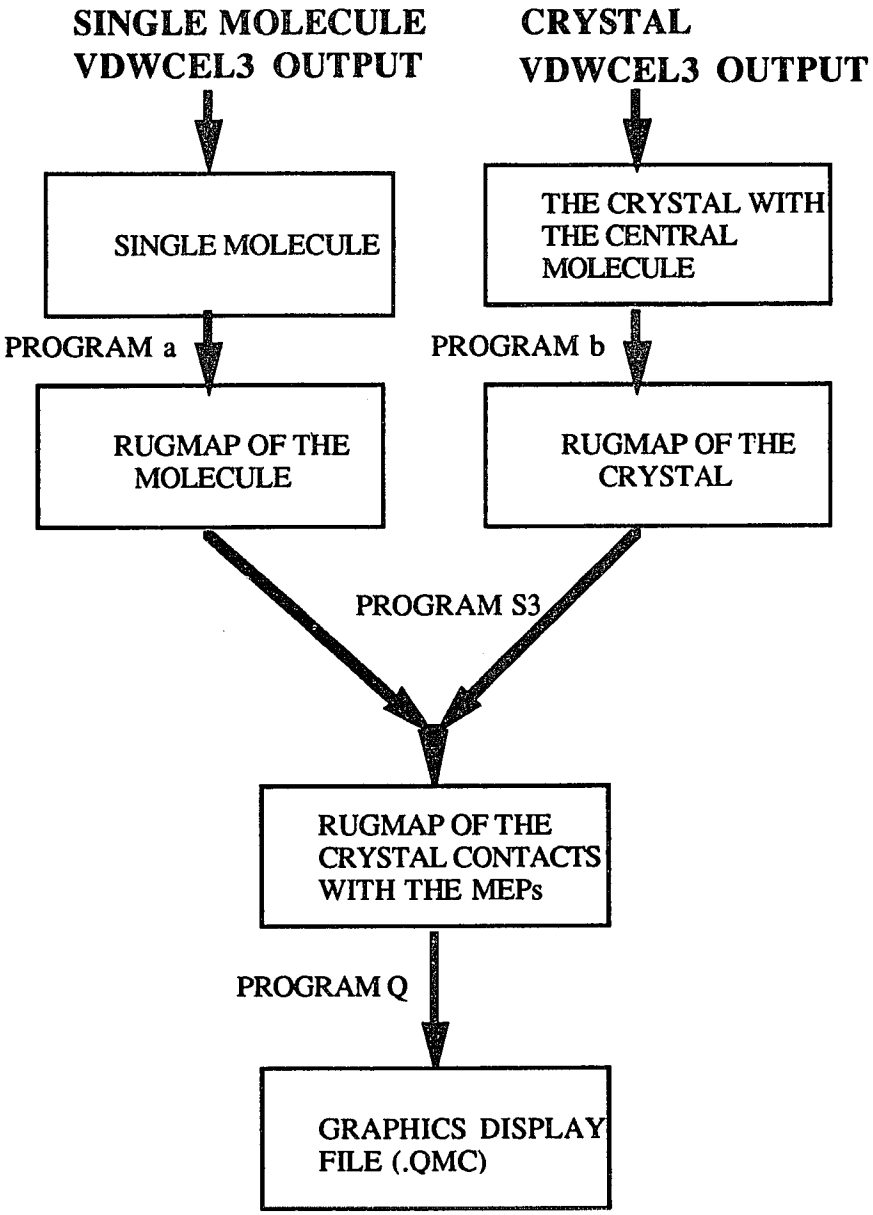
THE ANALYSES OF THE STERIC AND ELECTROSTATIC POTENTIAL CALCULATIONS:

Several programs were written in order to analyze the extensive amount of data generated from the program VDWCEL3. These programs were necessary in order to analyze and display the large amount of data which is difficult to compare and contrast by simple observation alone. One benefit from this approach is that the programs which analyze the data are more objective and thorough than visual analysis alone. After

the data is transfered from the Vax 11/780 Computer to the graphics terminals, the results of the analyses are visualized as a three dimensional, color-coded surface.which are labeled rugmap files. They are called rugmap files because one two-dimensional grid appears like a rug when displayed on the graphics terminal. One caveat of this approach is that some potentially useful information may be lost from the selection criteria being too narrowly defined. In order to guard against this potential loss, various combinations of programs were applied and compared for compatible results.

The CHARGE and CELL output files from the program VDWCEL3 are the rugmap files which contain the MEP and steric volume information respectively for a particular molecule. The Fortran programs A and B read the CHARGE and CELL output files and create new files which contain dummy hydrogen atoms with the x,y,z coordinates from the VDWCEL3 output file plus the associated property such as the MEP or van Der Waals surface (steric) as the fourth parameter. This facilitates the conversion to the format which is necessary to display the rugmap on the Silicon Graphics Workstation running Polygen's Quanta Program (see figure 5). From the first, RMS superpositioning, a rugmap file is compared with the complimentary rugmap file from another molecule to find the regions of similarities and differences either for the MEPs or for the steric properties of the molecules.

Figure 5: Flow chart of the output from the program VDWCEL3 to show the relationships between the programs and the subsequent display of the data on the computer graphics terminal.



The comparisons for the congruent grids, or rugmap points, of the two molecules were generated by the program A1 which accepts as input two rugmap files. The prgram A1 generates the comparisons of the MEP and steric rugmap files which are output from the programs A or B by color coding congruent grid points according to the number of possible combinations for the three values which are possible for the MEP (positive, negative and neutral) or steric (occupancy of first molecule, occupancy of second molecule and occupancy of both molecules) properties. For a particular physicochemical property such as the MEP there are nine possible combinations (ie. 3 x 3: a positive with a negative, a positive with a positive, etc.). Each of the nine possible combinations are assigned a number for that region of space, color-coded and displayed on the graphics terminal. This program together with the programs that read the output from the program VDWCEL3 provides a three-dimensional representation of regions of similar, different and neutral MEPs or the steric volume differences for two molecules. Other programs (N1 and C1) were written in order to compare and contrast the physicochemical properties for more than two molecules. These programs were necessary for the comparison of one experimental class of molecules with another experimental class of molecules.

The amount of information which is generated by these programs quickly becomes difficult to interpret visually for several large molecules, therefore, data reduction programs are important for analyzing and processing of the information for a visual display. The

regions of equality for any two files output from any of the programs were determined by the program T1 which saved these regions of equality for display on the graphics terminals. Similarly, the regions of difference between any two files output from any of the programs were determined by the program D1 which produced two difference files, each containing the regions where one molecule differed with the other molecule. These programs were important in simplifying the visual display of a large amount of data. A brief description follows each of the programs given in the list (Table I).

The basic complimentary regions of the MEP surface which the central molecule has within its crystal environment were determined by the programs S1 and S2. The regions which are negative in the crystal environment are expected to match those regions which are positive in the central molecule and vice versa. The programs S1 and S2 found and saved for susequent display, the complimentary regions which a molecule makes with its crystal environment.

For the comparisons of the two substrate molecules with the other experimental classes of molecules the Fortran program N1 was written in order to accept two files as input, template files with which to compare a third file. The input files were the steric files from the program B. The program N1 generates and color codes the eight possible steric combinations. There are eight possible combinations because each molecule has either a presence or absence of the van der Waals region which is compared for each of the three molecules (2 x 2 x 2 = 8) for a total of eight possible combinations.

Analogous to the program N1, the program C1 was written to accept two MEP files from the program A as input and compare these files to a third MEP file from another molecule from a separate experimental class. This was done in order to generate and color code the possible combinations of MEPs (for one molecule the MEP can be either, positive, neutral or negative). This is analogous to N1 described above, but in this instance, for three different molecules, the number of possible combinations is twenty seven for the MEPs (3 x 3 x 3 = 27). This becomes unwieldly to color code and visually inspect for important regions of similarities and differences; therefore, programs were designed to search for these similarities and differences and write the output in a format which was suitable for display on the graphics terminals.

Table I: A List of the Programs which were written for this thesis.

NAME	A BRIEF DESCRIPTION OF THE PROGRAM
1) A.FOR	Converts CHARGE.DAT Files from VDWCEL3 to altered RUGMAP Format in which the charges (RVALS) are associated with an atom name (H) and coordinates (MEP RUG FILE)
2) B.FOR	Same as A.FOR except this program takes CELL.DAT FILES from VDWCEL3 and where A.FOR reads an RVAL=77 and converts it to a -10.000, B.FOR reads an RVAL=99 and lists it as 99.000
3) A1.FOR	Takes as input two rugfiles from A.FOR and analyzes the nine possible regions of electrostatic potential interactions.
4) B1.FOR	The same as A1.FOR, except the analysis is the four possible combinations for the steric analysis
5) T1.FOR	Takes as input rugfiles from the other programs and finds the RVALS which are equal and converts the RVALS which aren't equal into zeros.
6) PN.FOR	Takes as input any rugfiles and prompts the user to select a specific RVAL to selectively prune for and retain. Other RVALS are turned into zeros.
7) Q.FOR	Prunes any rugfile of the zeros.
8) S1.FOR	Accepts as input a steric rugfile made by B.FOR and the rugfile made by A1.FOR to find the complimentary regions of electrostatic potential between the central molecule and its local environment.
9) S2.FOR	Same as S1.FOR, but doesn't remove zeros.
10) N1.FOR	Takes as input three steric rugfiles from B.FOR and compares the regions of the first two rugfiles with the regions of the third.

NAME	A BRIEF DESCRIPTION OF THE PROGRAM
11) C1.FOR	Takes as input three rugfiles made from A.FOR and compares two of the files with the third to analyze the electrostatic potential regions which are in common for these regions in one or more of the molecules.
12) D1.FOR	Performs the opposite function of T1.FOR. The program finds the regions of inequality between two molecules and prints out these regions of inequality into separate files for the two molecules.
13) D2.FOR	Accepts as input the output file from A1.FOR and the two rugfiles. The regions of extreme difference (ie. RVALS= 1 or 2 in A1.FOR output) are found and printed out as new RVALS.
14) PN1.FOR	Similar to PN.FOR, but prunes the RVALS which are between the positive and negative values of the selected RVAL.
15) EPAD.FOR	Takes as input two MEP rugfiles and prompts the user to choose either subtraction or addition of the files and then writes out the appropiate file.
16) S3.FOR	Written to take STERIC and MEP files, find the regions which have steric overlap (99s) and read the corresponding MEPs from the MEP file.
17) SUMMA-BAT3	Written to accept the rugmap files of the crystal contacts with the associated MEPs. Successive orientations are calculated and the lowest sum of the absolute differences of the MEPs is stored with the position of the molecule.

OUTPUT ANALYSIS AND REDUCTION:

Because the quantity of output is too large for visual inspection alone, data reduction techniques were coupled with the computer programs which analyzed and reduced the information for a display of the output which was easier to view and interpret. These programing techniques for data reduction required interfacing the Fortran program output from the VAX 11/780 Computer to the graphics display on the Silicon Graphics Computer running Polygen's Quanta Program. Several of these programs which were written for the VAX Computer also performed the analyses of two or more rugmap files.

The reliance upon programing for data reduction and analysis allowed for standardization of these processes. The standardization which was used for this thesis was first to identify the identical elements within the experimental class of molecules and then to use those identity elements of the class to find the elements which were similar or different between one experimental class of molecules and another experimental class. Various Fortran programs were written to accomplish this task. The Fortran program PN inputs any of the files in rugmap file format and then selects for a specific value as a fourth parameter which is pruned from the original file and stored in a new file. The selected value will be the only value retained in the new file for display. The program PN1 is similar to the program PN, but selects for a range of values which are between selected positive and negative values for display. The Fortran program Q selectively prunes zeros

from the fourth parameter column which facilitates both the manipulation and display of the data reduction files. After these data reduction techniques, identification can be made easily for those regions which display the similarities or differences for the experimental classes of molecules with pyroglutamyl peptidase II.

Although these programs allowed for some flexibility in the choice of values to display, the programs were applied equally to each experimental class of molecules. Only those regions of exact identity were accepted as belonging to an identity set for a particular class of molecules. For instance, if the MEP at a particular grid point was -5 for one molecule within a class, but -4 for another molecule, these were not accepted as identical regions. This could result in a too stringent definition for identity within a particular class, however, it minimizes the risk of accepting nonidentical elements. Neutral or zero valued elements were routinely extracted before displaying the physicochemical data with the molecule. Regions of differences were choosen as opposites. For example, either occupancy or vacancy for a steric difference and either positive or negative for a MEP difference (a positive with a neutral was not included as a difference for the MEPs within these criteria.

EXPERIMENTAL RESULTS:

Most of the experimental results were previously published (Lanzara et al., 1989) and are included in Appendix I. Representative Dixon plots for the determinations of the apparent inhibitor constants, K_is, are shown in Figures 6, 7 and 8 for TRH, analog II and analog V. At least five concentrations of the inhibitor were used for two different substrate concentrations. Each determination displayed is the best result from at least three separate experiments which were carried out with different preparations of the enzyme.

The inihibition constants for the substrates, which were determined by HPLC, are only the apparent inhibition constants. Because the substrates have the additional k_3 (or k_{cat}) term, the apparent inhibition constants appear larger than the simple dissociation constants for the other molecules which are not metabolized by PPII. This observation, however, does not appreciably change the rank order of inhibition for these molecules nor the subsequent conclusions from the experimental work. The experimental results are summarized in the table at the end of this section.

Figure 6: The Dixon plot for TRH with PPII.

The measured substrate was the naphthylamide derivative of TRH, TRHNA which was incubated with PPII in a coupled assay (Friedman & Wilk, 1986) with varying amounts of TRH as the apparent inhibitor. This is a representative plot from three separate experiments which included different preparations of the enzyme, PPII.

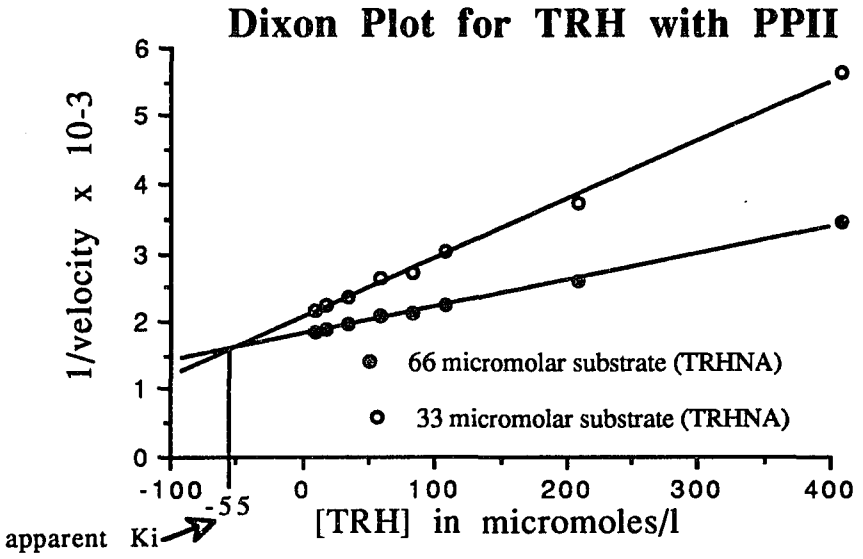


Figure 7: The Dixon plot for analog II with PPII.

The measured substrate was the naphthylamide derivative of TRH, TRHNA which was incubated with PPII in a coupled assay (Friedman & Wilk, 1986) with varying amounts of analog II as the apparent inhibitor. This is a representative plot from four separate experiments which included different preparations of the enzyme, PPII.

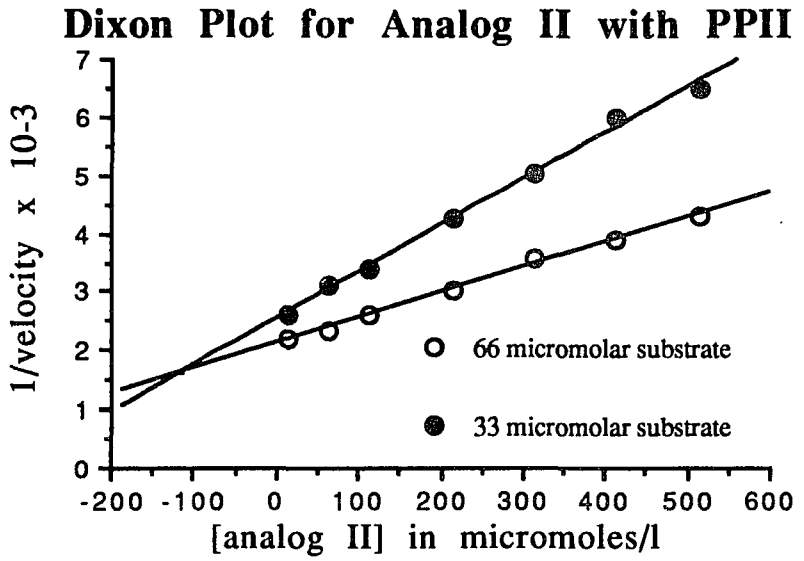
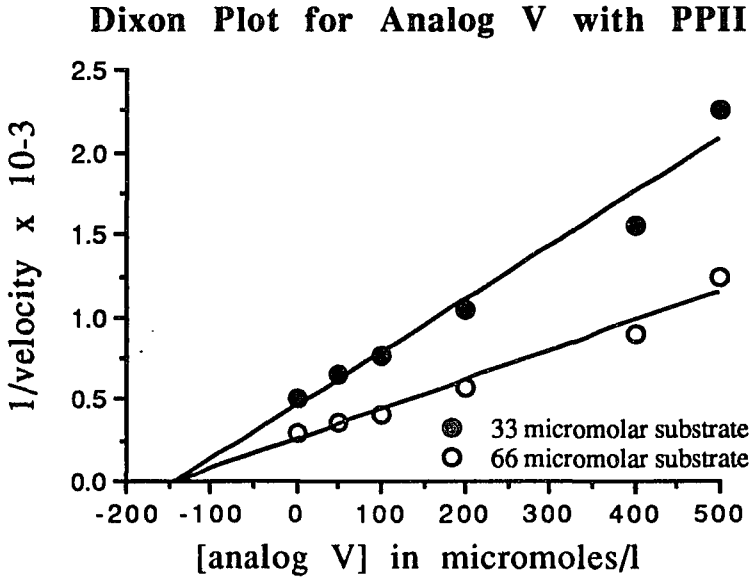


Figure 8: The Dixon plot for analog V with PPII.

The measured substrate was the naphthylamide derivative of TRH, TRHNA which was incubated with PPII in a coupled assay (Friedman & Wilk, 1986) with vaying amounts of analog V. This is a representative plot from three separate experiments which included different preparations of the enzyme, PPII.



In Figures 9 and 10, are the Cornish-Bowden plots for TRH and analog V. The Cornish-Bowden plots were compared with the Dixon plots in order to determine the type of inhibition in an unambiguous way (Dixon et al., 1979).

Figure 9: The Cornish-Bowden plot for TRH with PPII.

This is an alternative plot of the data from Figure 6. This plot is the substrate concentration, (TRHNA) divided by the velocity verses the concentration of TRH. Taken together with the plot in Figure 6 (Dixon et al., 1979), this plot shows that TRH is a competitive inhibitor of PPII.

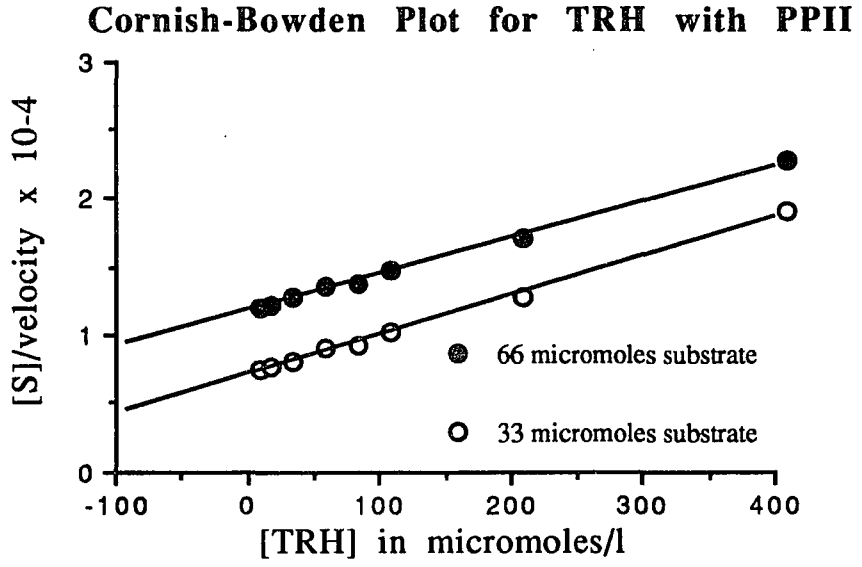
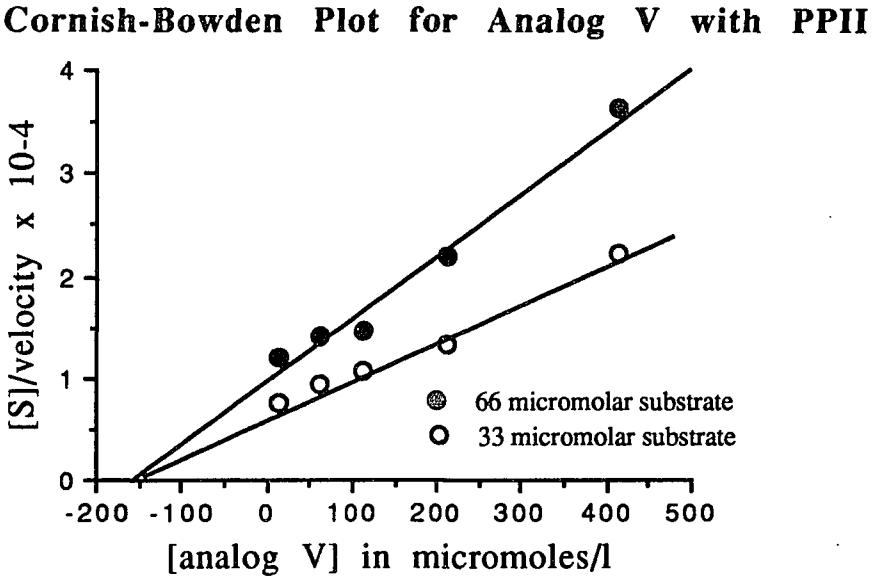


Figure 10: The Cornish-Bowden plot for analog V with PPII.

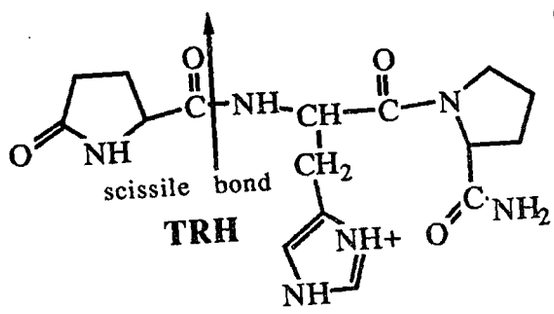
This is an alternative plot of the data from Figure 8. This plot is the substrate concentration (TRHNA) divided by the velocity verses the concentration

of analog V. Taken together with the plot in Figure 8 (Dixon et al., 1979), this plot shows that analog V is a noncompetitive inhibitor of PPII.

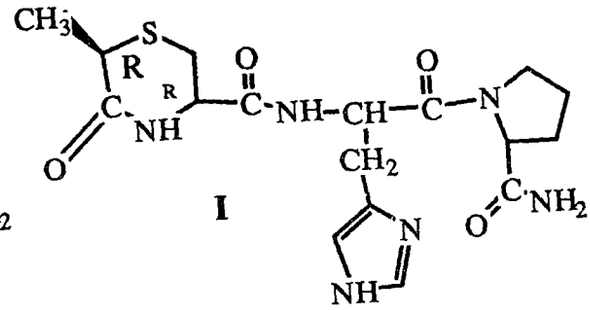


The values of the apparent K_is of the analogs ranged from 82 to 652 μM. These values compared to the values for the K_m of TRH-NA (52 ± 11 μM) and the apparent K_i of TRH (55 ± 8 μM) suggest that the analogs studied have less affinity than TRH for PPII. The rank order of potencies for the apparent K_is of the ten molecules which were tested with PPII is: **TRH > DN-1417 > MK-771 > III > PS-52 > II (S diastereoisomer of CG-3703) > VII (Phe-TRH) > V > VI > I (R diastereoisomer of CG-3703).**

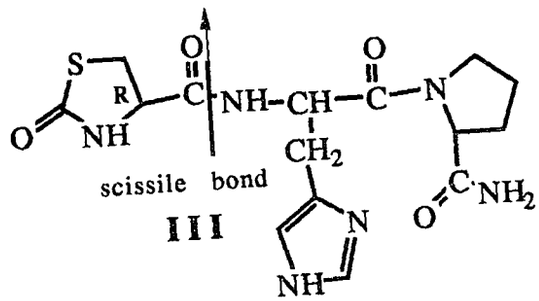
Figure 11: On the next page are the structures of the ten molecules which were experimentally studied with PPII.



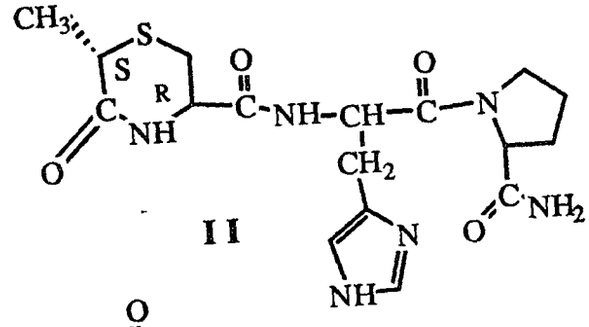
TRH



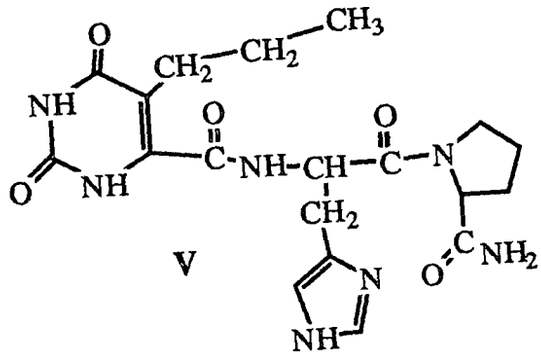
I



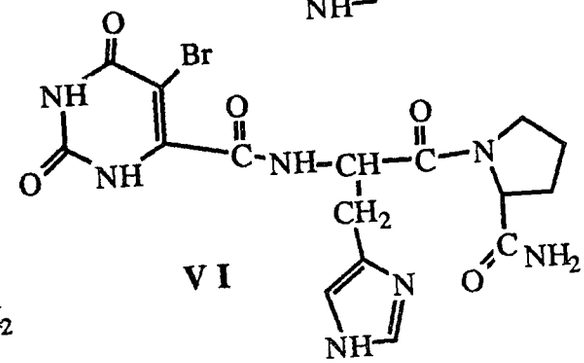
III



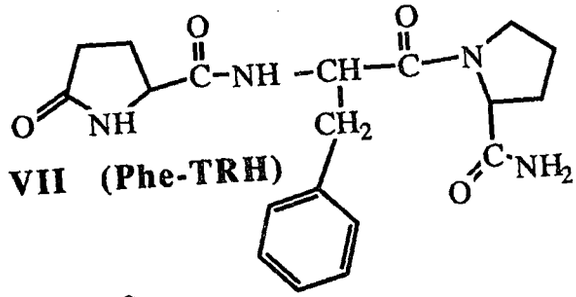
II



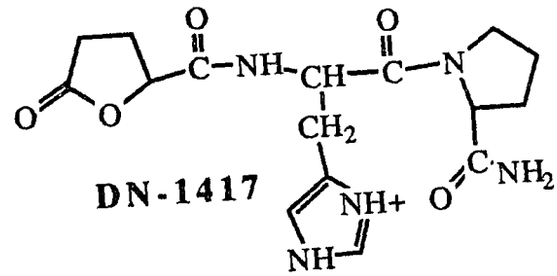
V



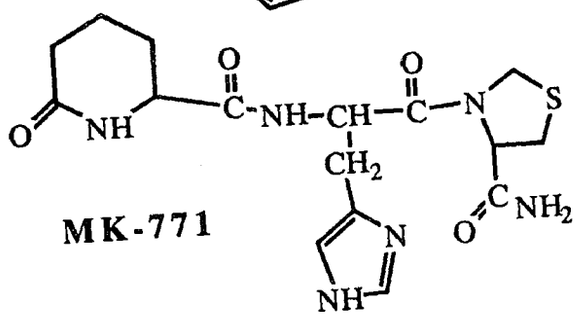
VI



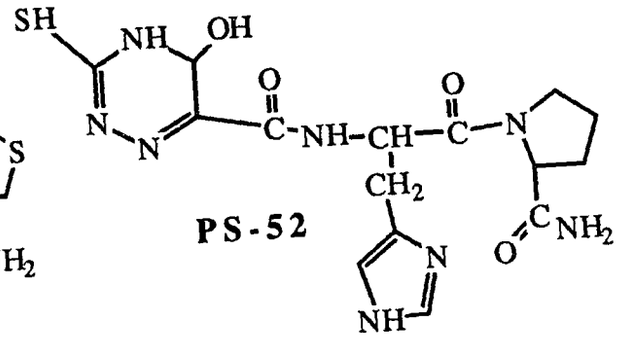
VII (Phe-TRH)



DN-1417



MK-771



PS-52

Table II: Summary of the experimental data obtained for the ten molecules with PPII.

NAME	CHEMICAL NAME	MW	Ki (μM)	TYPE OF INHIBITION	CLEAVED
1) TRH	L-pyroglutamyl-L-histidinyl-L-proline amide	362.4	55 ± 8 (2)*	COMPETITIVE	yes (100%)
2) I (CG 3703)	(3R,6R)-6-methyl-5-oxothiomorpholin-3-ylcarbonyl-L-histidinyl-L-proline amide	409.5	652 ± 176 (5)	COMPETITIVE	no
3) II (CG 3703)	(3R,6S)-6-methyl-5-oxothiomorpholin-3-ylcarbonyl--L-histidinyl-L-proline amide	409.5	166 ± 46 (4)	COMPETITIVE	no
4) III	(4R)-2-oxothiazolidin-4-ylcarbonyl-D-histidinyl-L-proline amide	380.4	108 ± 70 (4)	COMPETITIVE	yes (130%)
5) V	5-n-propylorotyl-L-histidinyl-L-proline amide	431.45	202 ± 18 (3)	NONCOMPETITIVE	no
6) VI	5-bromoorotyl-L-histidinyl-L-proline amide	468.27	437 ± 109 (3)	NONCOMPETITIVE	no
7) VII (Phe2TRH)	5-oxo-L-prolyl-L-phenylalanyl-L-proline amide	372.45	187 ± 23 (3)	COMPETITIVE	no
8) DN-1417	γ-butyrolactone-γ-carbonyl-L-histidinyl-L-proline amide	555.49 (citrate salt)	82 ± 25 (3)	COMPETITIVE	yes (129%)
9) MK-771	L-N-(2-oxopiperidine-6-carbonyl)-L-histidinyl-thiazolidine-4-carboxamide	394.454	87 ± 26 (3)	COMPETITIVE	no
10) PS-52	3-sulfhydral-5-hydroxy-1,2,4-triazine-L-histidinyl-L-proline amide	408.436	143 ± 46 (2)	NONCOMPETITIVE	N.D.

* the number in parentheses refers to the number of experiments
N.D. = not determined

Three of the analogs, V, VI and PS-52 displayed noncompetitive enzyme kinetics (Table II). A structural similarity among the analogs which displayed noncompetitive kinetics is the planar, sp2,

configuration of the alpha-carbon adjacent to the carbonyl of the orotyl rings which are substituted for the pGlu ring of TRH (see Figure 11 above). At first glance, PS-52 with the triazine ring doesn't seem to fit with the other noncompetitive inhibitors, however, the alpha-carbon adjacent to the carbonyl has an sp2 configuration and the electronegative groups of the ring correspond to the carbonyl oxygens of the orotyl rings if the triazine ring was rotated by 180°. A plot of the reaction velocity versus total enzyme concentration demonstrates that these noncompetitive inhibitors are reversibly bound (see Figure 12).

In Figure 13 is a graph of the relative peak heights for analog III and TRH with PPII verses time. The high pressure liquid chromotography (HPLC) tracings were used to determine the degradation of analog III relative to the degradation of TRH by PPII. Analog III was degraded in each case about 125 ± 25% relative to that for TRH by PPII for three separate experimental determinations which included different preparations of the enzyme.

Figure 12: A plot of Vmax verses the amount of total enzyme.

This plot distinquishes between a reversible and irreversible noncompetitive inhibitor (Segel, 1975). The plot shows varying amounts of PPII in the presence and absence of 250 μM of the noncompetitive inhibitor, analog V. Incubation conditions were the same as for the inhibition assays as described in the methods above.

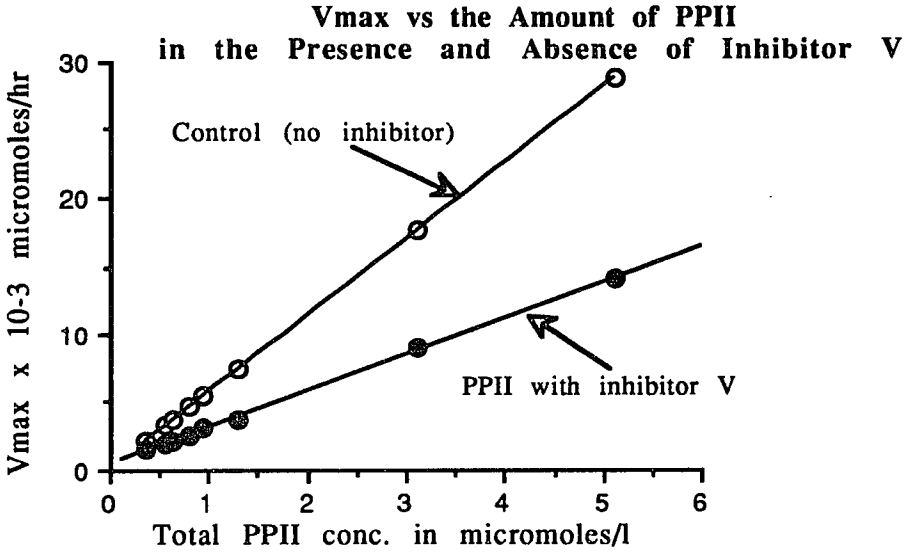
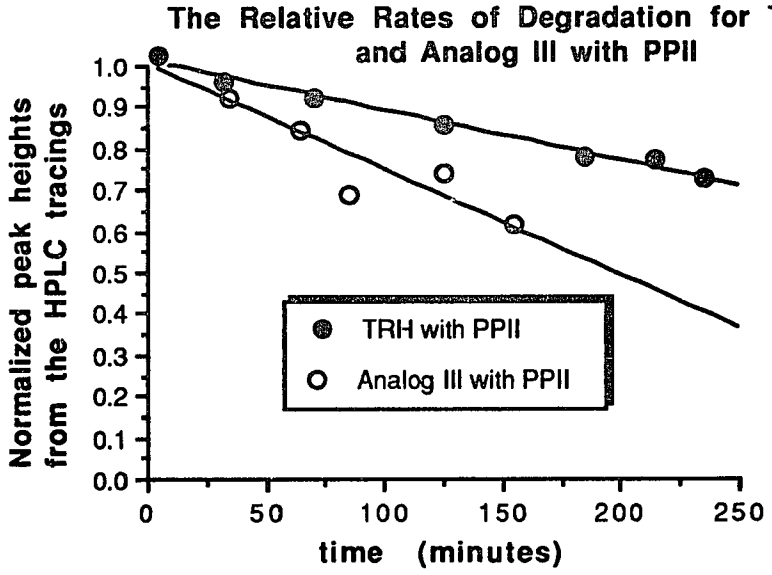


Figure 13: The relative rates of degradation for analog III and TRH with PPII.

Degradation of the analogs was monitored by HPLC. The assay was performed with 20 to 40 µl of a 10 mM stock solution of the molecule to be tested with 50 to 100 µl of the PPII enzyme preparation (or buffer only for the control) with enough TRIS/HCl buffer to bring the final volume to 400 µl. The samples were incubated in a water bath at 37° C. Incubation mixtures contained the enzyme, PPII, a molecular analog of TRH and the Tris-HCl buffer. At various time intervals, a micropipette was used to withdrawal 10 µl samples and inject them into a C18 reverse phase column at varying time intervals. The solvent system for the column was an isocratic gradient of 10% methanol in a 0.05 M potassium phosphate buffer at pH 5.0. Elution tracings were calibrated with a known solution of cycloHis-Pro and TRH or the analog molecule.



Since DN-1417 was unstable in solution, only a freshly made solution was used to determine the value of the K_i and to perform the HPLC studies. The degradation of DN-1417 was similar to that for analog III, but was complicated by its tendency to spontaneously decompose into four separate peaks. Only one of the peaks subsequently decomposed in the presence of the enzyme, PPII.

From the HPLC measurements the replacement of the pGlu-ring with six-membered heterocyclic rings prevents degradation by PPII. Thus MK-771 is not cleaved and the Phe-TRH (analog VII) is also resistant to degradation suggesting that the replacement of histidine by phenylalanine prevents the hydrolysis by PPII. By contrast the five-membered ring, thiazolidinone analog, III, and the butyrolactone analog, DN-1417, are degraded more rapidly than is TRH by PPII.

From the previous experimental results, the analogs can be divided into four experimental classes; however, the molecules within the second experimental class (see Table III below) can be classified within either the third or the fourth classes as either competitive or noncompetitive inhibitors of PPII. None of the noncompetitive inhibitors were metabolized by PPII. In the first class are the analogs which were substrates for PPII (see Table I). These substrates appeared as apparent competitive inhibitors of PPII by the Dixon and Cornish-Bowden plots; however, the apparent K_is for these molecules include the k_{cat} term which causes the K_is to be larger than the corresponding dissociation constants for these molecules. In the second class are the analogs which were not cleaved by PPII. In the third class

are the noncompetitive inhibitors of PPII. And in the fourth class are the analogs which are the competitive inhibitors of PPII.

Table III: A summary of the experimental classifications for the ten molecules with PPII.

substrates	uncleaved molecules	non-competitive inhibitors	competitive inhibitors
TRH	I	V	I
III	II	VI	II
DN-1417	V	PS-52	Phe-TRH
	VI		MK-771
	Phe-TRH		
	MK-771		

COMPUTATIONAL RESULTS:

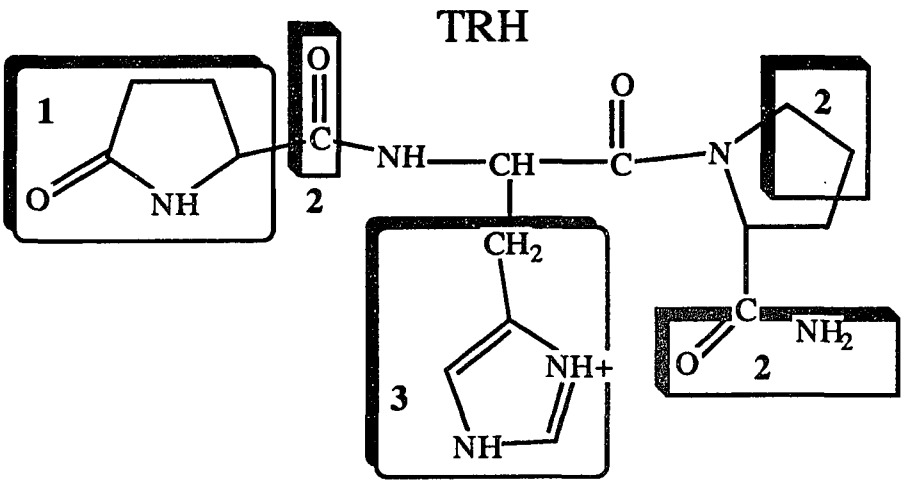
The results from the computational analyses will be presented as four separate categories. These categories are: 1) the partitioned distance matrix; 2) the superpositioning of the molecules by the minimization of the root mean square distance between atom pairs (RMS method), from the molecules to be superimposed, which encompass the scissile bond region; 3) the superpositioning of the molecules by searching for the smallest absolute difference between the molecular electrostatic potentials (MEPs) within the crystal contacts (CCs) of the crystal environment for each of the molecules (CC method) and 4) the placement of these new orientations within the active site of carboxypeptidase-A in order to observe the relative orientation of the molecules to the zinc atom and for the possible steric constraints within the active site. Two different superpositioning methods were used in order to look at different aspects of the molecules.

The first method, RMS method, is used to examine the similarity of all of the molecules within the region of the scissile peptide bond. This aspect is examined in order to see whether or not there exists some detectable differences between the molecules which are cleaved and those which are not cleaved. The second aspect is to examine the similarities and differences between the noncompetitive and competitive inhibitors. This aspect is examined in order to see to what extent the physicochemical properties of the noncompetitive inhibitors differ from those of the competitive inhibitors

1) the partitioned distance matrix: The program DISDIF3 creates the averaged signed (avesig) matrix and averaged absolute (aveabs) matrix from the partitioned distances of the intramolecular distances of analog III minus the corresponding, partitioned distances of another molecule. The aveabs matrices are analyzed for differences which correlate with the experimental data. The partitioned distance matrix (PDM) data can be associated with three of the experimentally observed properties of the molecules. These properties of the molecules are 1) the substrates, 2) the rank order of the apparent affinities of the molecules for PPII and 3) noncompetitive inhibition of PPII. Analog III serves as a template molecule which represents the substrates of PPII. From the analyses of these three experimental classifications of the molecules, specific regions within the PDM can be identified that associate with the experimentally observed properties of each class. These regions are summarized below and diagramed in Figures 14-16.

Three regions are found which associated with the cleavage of the molecules by PPII. The numbered regions are, beginning at the N-terminal, 1) the pyroglutamyl ring, 2) the pyroglutamyl carbonyl with a region of the proline ring and 3) the C-terminal amide and the histidyl ring (see Figure 14).

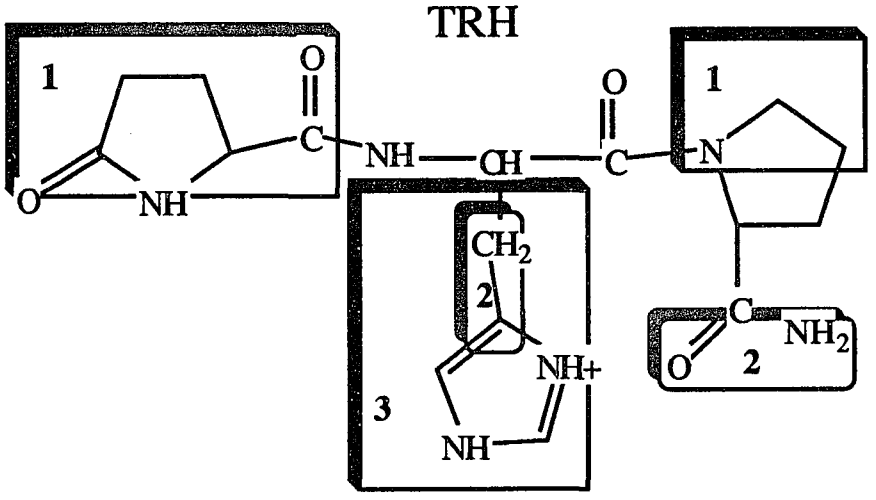
FIGURE 14: Regions which were associated with the cleavage of TRH and analog III by the PDM analysis.



Regions associated with the cleavage of TRH and analog III by PPII

Three regions are found to associate with the apparent affinities of the analogs for PPII. Beginning at the N-terminal of the molecule, the numbered regions are 1) the pyroglutamyl ring with a region of the proline ring, 2) the beta and gamma carbons of the histidine residue with the amide region of the prolineamide and 3) the histidine residue. The altered spatial relations between these regions of the molecules suggest regions which are important for the apparent affinities of the molecules. However, the apparent affinities of the molecules could not be analyzed well due to the narrow range of affinities, which correspond to a free energy difference of only about 1.4 kcal (or about one order of magnitude difference between the K_is).

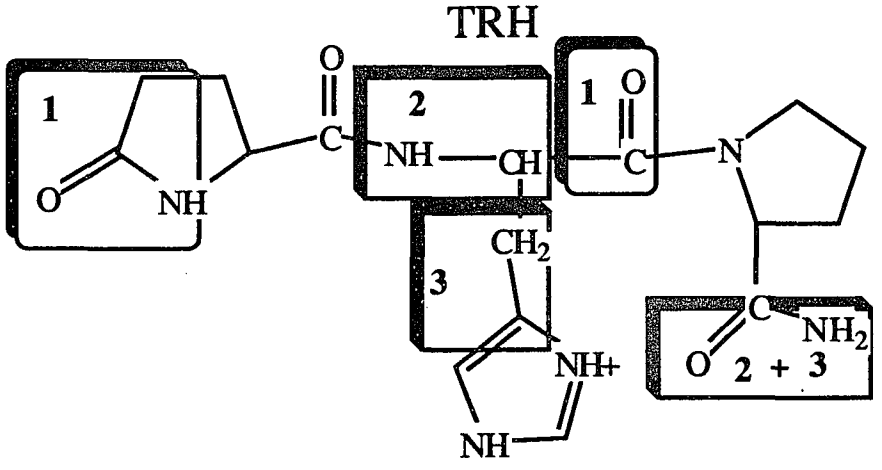
FIGURE 15: Regions which were associated with the affinities of the molecules for PPII by the PDM analysis.



Regions associated with the affinity of TRH and analogs for PPII

Three regions are found which associate with noncompetitive inhibition of PPII. Beginning at the N-terminal of the molecule, the numbered regions are 1) the pyroglutamyl ring with the histidyl carbonyl, 2) the alpha carbon and NH terminus of the histidine residue with the amide region of the prolineamide and 3) the beta and gamma carbons of the histidine residue with the amide region of the prolineamide residue (see figure 16).

FIGURE 16: Regions which were associated with the noncompetitive inhibition of PPII by the PDM analysis.



Regions associated with the noncompetitive inhibition of PPII

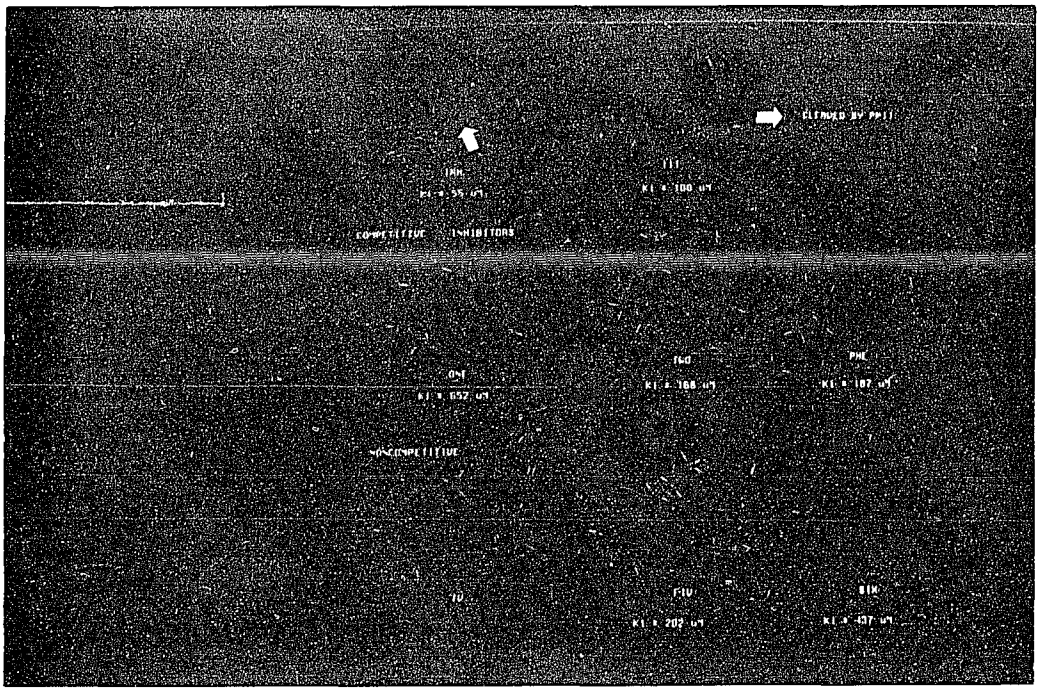
The differences within each of the regions above is not large enough to discriminate between the noncompetitive inhibitors (NCIs)

and the competitive inhibitors. Only when all of the regions are taken together as the total sum is there a noticeable difference.

2) Superpositioning of the molecules by minimization of the rms distance of the atoms which are located within the scissile bond region (first superpositioning - the rms method): The structural superpositioning of the six atoms (the four atoms of the peptide bond with the two neighboring C_α atoms), which are within the scissile peptide bond region, is done in order to compare the substrate molecules with those molecules which are competitive inhibitors but not cleaved. Analog III is compared with TRH because these two molecules are both cleaved by PPII. Analogs I, II and VII (Phe-TRH) were competitive inhibitors but not cleaved by the enzyme.

The crystal structure for TRH as compared to the other molecules is quite different. TRH was crystallized in the protonated form with the tartrate anion as the counterion (Kamiya et al., 1980) whereas all of the other analogs were crystalized as uncharged molecules in a mixture of propanol and water (Eckle & Stezowski, 1985). This may cause the criteria here to be overly restrictive, however, from the rather broad pH-dependence of the enzyme (7.0 to 7.5)(Wilk and Wilk, 1989b), the protonation state of TRH appears to matter little for its reactivity with the enzyme. This suggests that the criteria may be appropriate for the subsequent analyses.

FIGURE 17: The display of each of the x-ray crystal structures for TRH and the analog molecules after the first, RMS, superpositioning.



Beginning at the closed amino terminus of the molecules, the first noticeable difference is that the pGlu ring of TRH is rotated through the ψ torsional angle by 144° compared to the oxothiazolidin ring of analog III. Similarly, the histidyl ring of TRH is rotated through a torsional angle of 123° around the C_α-C_β bond compared to the histidyl ring of analog III. One reason why this conformation for TRH is more stable is the presence of an intramolecular hydrogen bond between the imidazolium cation and the oxygen from the prolineamide carbonyl group of TRH. The intramolecular hydrogen bond distance is 1.67 Å

from the hydrogen atom on the N1 atom of the histidyl ring to the oxygen atom on the C-terminus, and forms a ten membered ring which includes the two, His-Pro-NH2, residues of TRH. Analog III does not have this intramolecular hydrogen bond which is most likely due to the conditions of crystalization. However, it is not possible to test this assumption without a crystal structure for analog III which is crystallized under the identical conditions for TRH.

Because analog III is cleaved by PPII and crystallized under conditions which are similar to the other analogs, analog III is choosen as the template for comparisons with the other analogs. This allows comparisons with the previous results from the PDMs above for which analog III served as the template molecule.

III vs TRH: The major difference between analog III and TRH is the torsional rotation of the pGlu and histidyl rings. The rotation of the pGlu ring through the ψ torsional angle of 144° superimposes the N-terminal rings of TRH and analog III. Similarly, a rotation through 123° around the C_α-C_β bond superimposes the histidyl rings of TRH and analog III.

III vs Phe-TRH: Comparing analog III with Phe-TRH, the first noticeable difference is that the pGlu-ring of Phe-TRH is rotated through through the ψ torsional angle of 117° compared to the oxothiazolidin ring of analog III. If the phenyl ring of Phe-TRH is rotated through a torsional angle of 108° around the C_α-C_β bond, the phenyl ring aligns with the histidyl ring of analog III, the planes of the rings are almost at right angles to each other. Also, the amide of the prolineamide points

toward the pi cloud of the phenyl ring with nitrogen to carbon distances of 3.35 to 3.99 Å and the closest N-H to ring carbon distance being 2.44 Å. This appears to be similar to the hydrogen bond accepting behavior for the phenyl ring as described by Levitz and Perutz (Levitz and Perutz, 1988).

III vs I: The comparison of analog III with analog I shows that the N-terminal rings are close together within parallel planes. Analog I is a diasteromer of analog II with the methyl group of the oxothiomorpholin ring inverted from its configuration in analog II. The atoms which are farthest apart within the N-terminal ring of analog I compared to analog III are the carbonyl oxygens which are 1.0 Å apart and the sulfur atoms which are 0.71 Å apart. The nitrogen atoms of the rings are only 0.14 Å apart. However, the histidyl ring of analog I is displaced 1.29 Å within the plane of the histidyl ring of analog III compared to the 0.95 Å displacement for the histidyl ring of analog I. The proline rings are apart. The largest distance of the comparable atoms within the proline rings is 0.93 Å for the alpha carbons of the rings. The amide nitrogens are 1.13 Å apart, but the oxygens are 0.39 Å apart.

III vs II: The comparison of analog III with analog II shows that the N-terminal rings are near each other and parallel. Since analog II has the six-membered, oxothiomorpholin ring whereas analog III has the five-membered, oxothiazolidin ring in place of the pGlu of TRH, there are atoms which extend farther in the plane of the ring for analog II compared to analog III. The histidyl ring for analog II is displaced

within the plane of the histidyl ring for analog III. The imadazolium nitrogen to nitrogen distance for the two rings is 0.95 Å. The nitrogen of the amide of the prolineamide for analog II is reversed when compared to the nitrogen of the amide for analog III.

III vs IV: Beginning with the first or altered N-terminal pyroglutamyl ring, the planes of the rings are at right angles with the ethyl group from analog IV oriented near to the sulfur and carbonyl oxygen of analog III. The histidyl ring for analog IV is tilted about 20° from the plane of the histidyl ring of analog III at a distance of 1.21 Å. The proline peptide carbonyl oxygens are 0.53 Å apart. The planes of the proline rings are tilted at a 20° angle relative to each other. The distances between the atoms of the proline rings ranged from 0.83 Å for the ring nitrogen to nitrogen distance to 1.63 Å for the Cβ of the ring. The amide groups were reversed and 1.48 Å apart. Although analog IV was not tested with PPII, it is predicted to be a noncompetitive inhibitor of the enzyme since it has almost identical characteristics to analog V.

III vs V: The major difference between analog III and analog V is that the sp2 configuration of the alpha carbon in the N-terminal ring of analog V doesn't allow a matching orientation of the rings by torsional angle changes. This means that the N-terminal ring of analog V is orthogonal to the orientation of the N-terminal ring of analog III. The histidyl ring of analog V is tilted 21.39° out of the plane of the histidyl ring of analog III. The imadazolium nitrogens are separated by 1.27 Å between the two histidyl rings of analogs III and V. The amide

nitrogen of analog V points in the opposite direction compared to that for analog III.

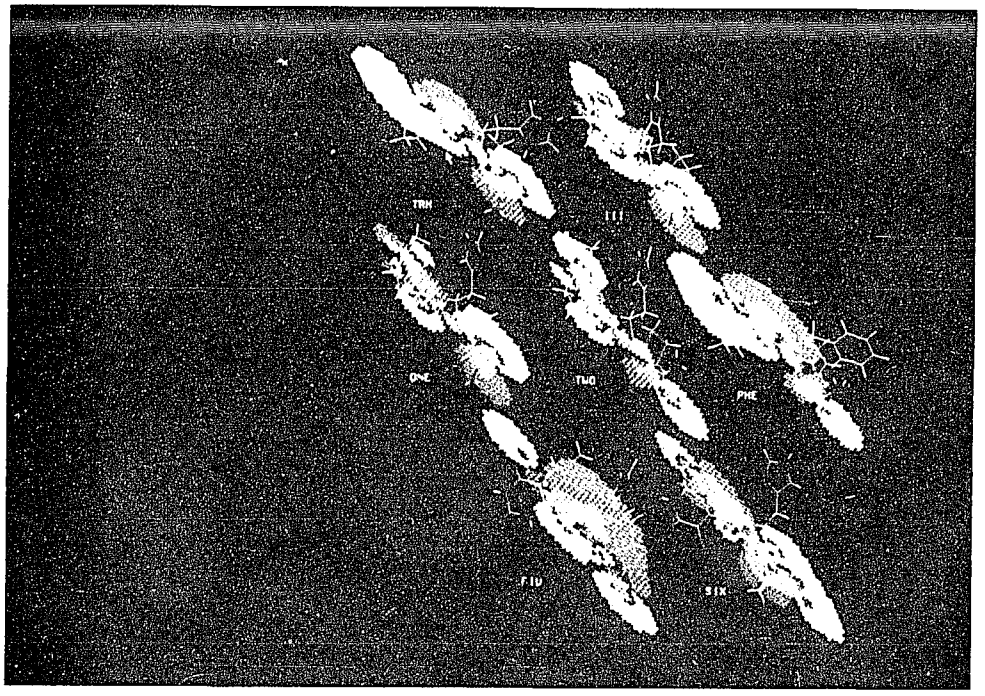
III vs VI: The planes of the N-terminal rings are at about 90° angles. The bromine atom of analog VI is oriented opposite to the sulfur atom of analog III. The histidyl ring for analog VI is flipped relative to the ring for analog III. The angle of the planes made by the two histidyl rings is 44.78°. The proline peptide carbonyl oxygens are 2.02 Å apart. Analog VI has a cis configuration for the proline ring whereas the other analogs have trans configurations. The distances between the nitrogen of the proline ring of analog III and the nitrogen of the proline ring of analog VI is 1.25 Å. The Cβ distance is 3.41 Å. The amide groups are reversed and rotated so that the oxygen atoms are 1.99 Å apart.

After the first superpositioning, the similarities and differences of the various molecules may be examined near the region of the scissile peptide bond. This region is examined in order to see whether or not there exists some detectable differences between the substrates and the molecules which are not cleaved, but are competitive inhibitors of PPII.

The computer program, VDWCEL3, calculated the molecular electrostatic potentials (MEPs) for a 24.5 by 22 by 24 Å box which includes all of the atoms for each of the molecules plus 6 Å on each side. In addition to the comparison of the spatial positions of the atoms, the color coded MEPs are displayed for their comparisons as well. These are displayed in figure 18 for each of the seven molecules through the plane of the scissile peptide bond. These MEPs calculated by the

program VDWCEL3 were compared to the MEPs calculated by the QUANTA program. In each case there was good agreement between the MEPs calculated by the VDWCEL3 program or the QUANTA program.

FIGURE 18: Display of the color coded MEPs through the plane of the scissile peptide bond for all of the molecules with x-ray crystal structures.



III vs TRH: TRH has two main regions of the MEPs, the first is the negative MEP regions through the plane of the scissile bond (which is marked with an arrow in Figure 18) which are colored white-light yellow to red depending on the magnitude of the negative MEP. The second is the positive regions which are colored purple and light blue

depending on the magnitude of the positive MEP. Analog III has a similar pattern to TRH with some small differences. This is consistent with the experimental evidence that analog III is cleaved by PPII.

III vs Phe-TRH: Phe-TRH has an essentially similar pattern within the pGlu region except that both the positive and negative regions appear magnified. Within the region of the prolineamide ring there appears a different pattern. There are a reversal of the negative and positive potentials within the prolineamide region. This may be consistent with the experimental data which shows that Phe-TRH is not cleaved by PPII.

III vs I: Analog I displays an increase of the positive potential in the pGlu region as well as a sharp reduction in the size of the negative potential. The prolineamide region, however, appears very similar to TRH and analog III.

III vs II: Analog II displays a negative potential within the pGlu region which is similar to this region of analog III. However, the prolineamide region has a ninety degree shift of the positive and negative potentials.

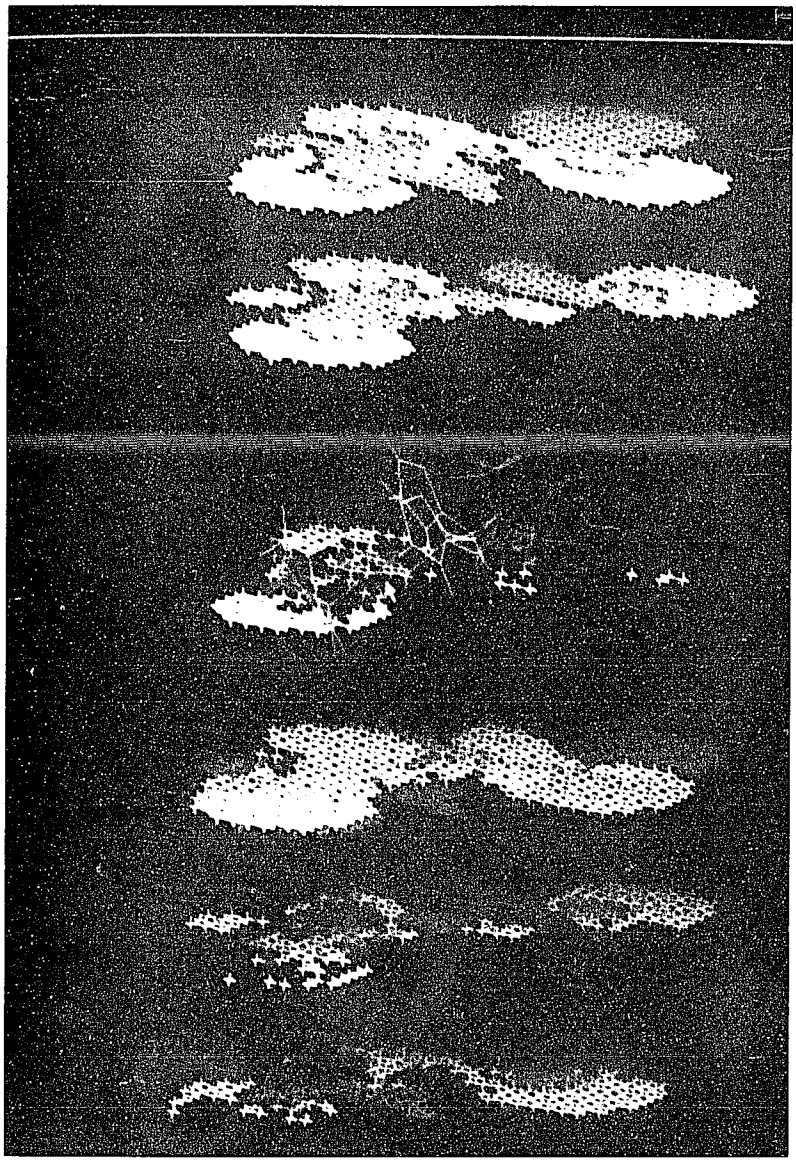
III vs V: Analog V has a more extensive positive region which extends from the pGlu ring region to the prolineamide region. This region of positive potential reverses the positions of the positive and negative potentials when compared to analog III.

III vs VI: Analog VI has a diminished negative potential and a heightened positive potential within the pGlu to His regions when compared with analog III. The prolineamide region shows similar

regions to analog III except the positive region has a larger magnitude and the negative region has a larger region of negative potential.

In order to examine the similarities and differences in more detail, computer programs were written to compare and contrast the steric and MEP maps from the VDWCEL3 programs. Initially only the plane through the scissile peptide bond was examined. The regions where the MEP was zero were not given a point (they were left blank for easier viewing). As seen for the comparisons of analogs II and III in Figure 19, there are many ways to display the information. At the top of Figure 19 is displayed the MEP of analog III and below that is the MEP of analog II. Below these are the stick structures for the two molecules

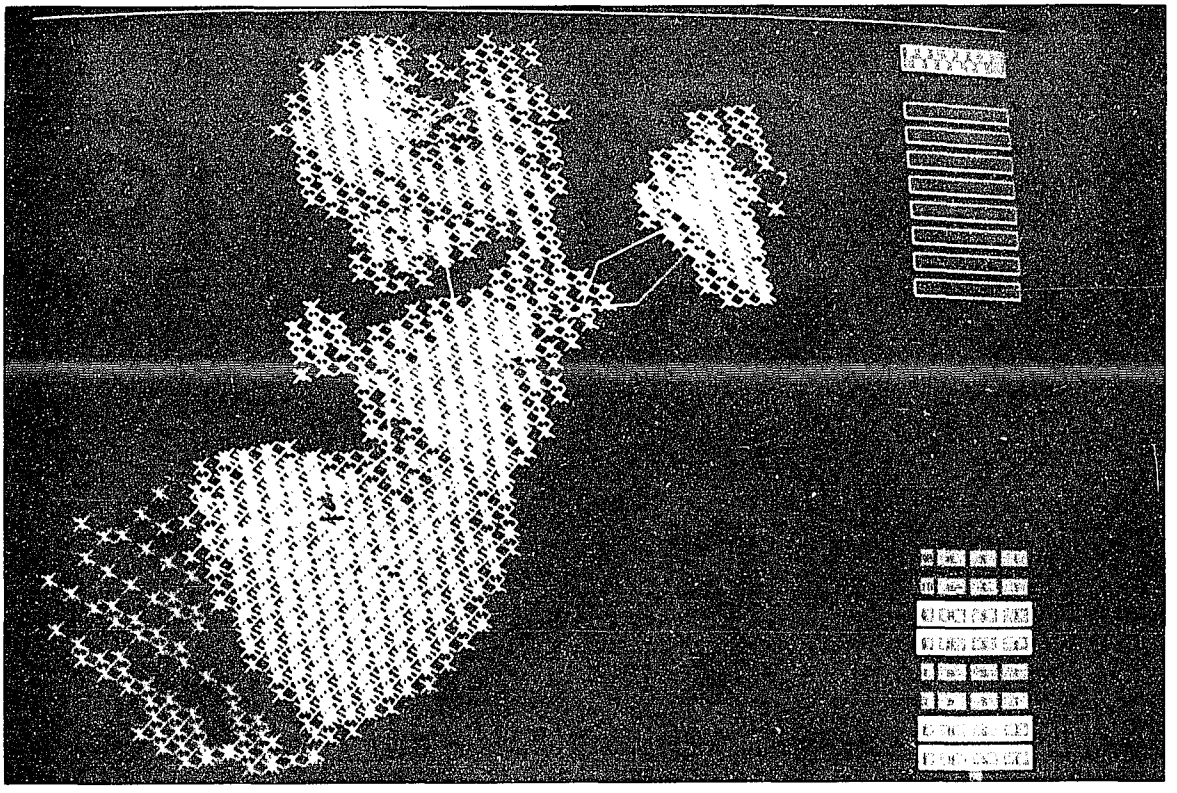
FIGURE 19: Analyses of the MEP maps for analog II compared with analog III.



with a map of those regions of the MEPs which are the same for the two analogs. Beneath that is the map of those areas analyzed by the program A1 which color codes and displays nine possible comparisons of the MEPs for regions of similarities and differences between the two molecules. Basically, the blue and purple regions represent those areas which are different in sign between the MEPs from the two maps and

the white and yellow colors represent those areas which are similar in sign between the two maps. The other colors such as red and green represent those regions where one or the other molecule's MEP is either a positive or negative, but the other molecule's MEP is a zero. Thus, some very fine distinctions can be made between the analogs two MEP maps. The next map displays those areas of any difference which analog II has with analog III and the sixth and last map displays those areas of any difference which analog III has with analog II. This type of analyses was done for the entire three-dimensional steric and MEP maps of the analogs. However, as seen in Figure 20, the three-dimensional representation of the areas of similarity (colored white and yellow) and difference (colored blue and purple) is overwhelming to compare visually. Certainly, it becomes impossible for comparing several molecules at once! Therefore, programs were written in order to calculate comparisons for the molecules.

Figure 20: The areas of similarity and difference between analog II and analog III.

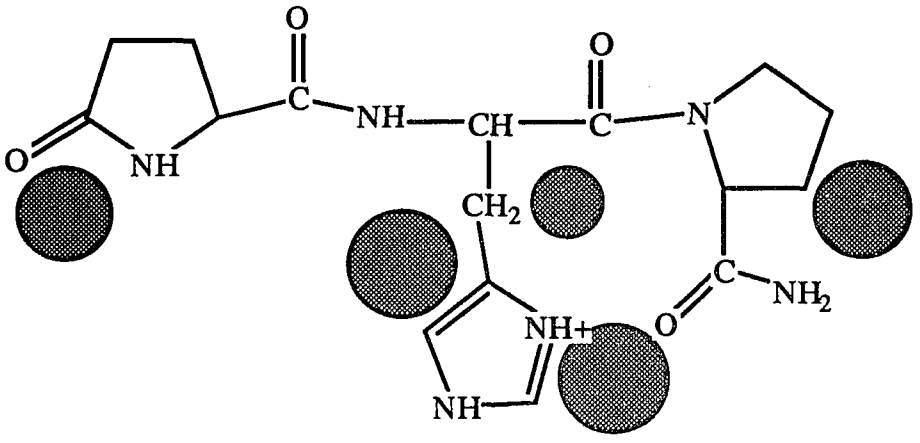


For the three-dimensional representations of the MEPs, the entire MEP for the isolated molecule is used for the comparison with the entire MEP from another molecule. All of the three dimensional regions in common for the molecules which are substrates are compared to the corresponding regions of the molecules which are not cleaved, but are competitive inhibitors of PPII. Only those regions which are identical in MEPs are accepted as regions of identity. Each of the competitive inhibitors, Phe, I, and II, are compared to the substrates, TRH and analog III. The information from each of these comparisons is analyzed by the computer programs, B, N1, T1 and PN, to find those regions

which are identical to the corresponding regions of the metabolized compounds and those regions which are different. The analysis shows that there are regions which are occupied by all of those competitive inhibitors which are not cleaved, but are unoccupied by both substrates, TRH and analog III (see Figure 21).

FIGURE 21: The steric regions, darkened circles, where the competitive inhibitors, analogs I, II and VII (Phe-TRH), are present but both substrates, TRH and analog III, are absent.

TRH



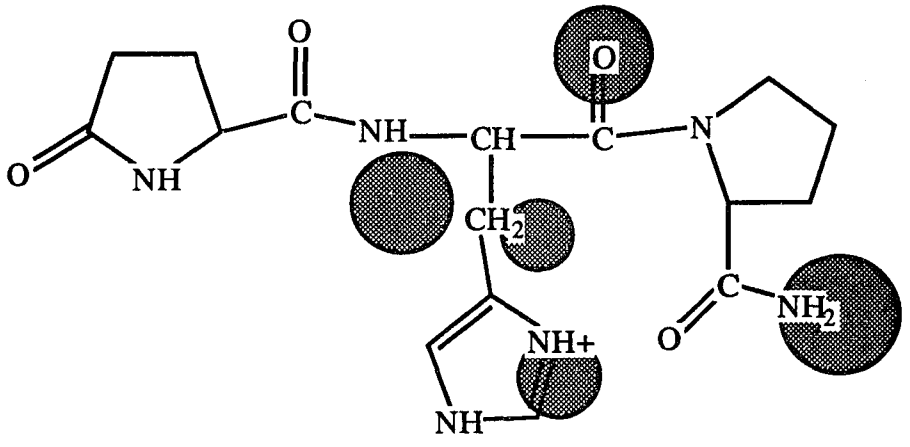
Regions where the other competitive inhibitors were present but both TRH and analog III were absent

This result is consistent with some of the known inhibitors (RX 74355 & RX 77368) which have methyl groups substituted in the region of the β carbon of the proline ring and are not metabolized (Griffiths et

al., 1989). However, the x-ray crystal structures are not available for these inhibitors at present, so the precise positioning of these groups relative to TRH and analog III would have to wait for the x-ray crystal structure determination. Similarly there are regions which are occupied by both TRH and analog III but remain unoccupied by all of those competitive inhibitors which are not cleaved (see Figure 22).

FIGURE 22: The steric regions, darkened circles, where both TRH and analog III are present, but the competitive inhibitors, analogs I, II and VII (Phe-TRH), are absent.

TRH



Regions where both TRH and analog III were present but the other competitive inhibitors were absent

This suggests that there exist some regions which require occupancy in order for PPII to cleave the molecule and that the occupancy of other regions will prevent the cleavage of the molecule by

PPII. The criteria which is used for this analysis may be too strict, since only an identical steric region is accepted as defining a common region between an analog together with both of the identical steric regions of overlap for both TRH and analog III.

There occur regions which are on or near the histidyl and pyroglutamyl rings and which can be associated with the cleavage or noncleavage of the analogs by PPII (see Figures 19 & 22). This is consistent with the results obtained from the PDM analysis above. Whether there are inhibitors which have groups in these positions and are not cleaved by PPII is not known at this time. This analysis may have to wait for further characterization and x-ray crystal structure determination.

The superpositioning results for the NCIs suggest that the rings of the NCIs are oriented 90° relative to the pGlu ring of TRH and that these NCI analogs may have different relative orientations than those of the competitive inhibitors or substrates.

3) Superpositioning by searching for the smallest absolute difference between the crystal contacts (second superpositioning - the CC method): After the initial, RMS, superpositioning, the program, LIMITS, calculated the consensus intervals for each of the superpositioned molecules (see Table IV). The consensus intervals include the largest x, y and z directions for each of the superimposed molecules. The consensus intervals are a necessary

guide for the subsequent comparisons of the crystal environments for each of the molecules.

TABLE IV: The results from the program LIMITS.

name	X axis:		Y axis:		Z axis:		CENTER	#
	min	max	min	max	min	max	(x,y,z)	ATOMS
TRH	0.46	8.75	4.41	13.32	-0.79	9.05	(4.3,8.5,5.0)	49
TRHCR	-12.00	19.27	-3.70	23.50	-9.05	16.43	(2.0,9.7,3.6)	1050
PHE	0.51	8.81	4.50	13.11	-0.78	10.78	(4.3,8.8,5.2)	51
PHECR	-11.65	23.02	-11.06	29.81	-22.69	27.49	(5.4,8.1,2.5)	1566
ONE	-2.97	8.64	5.79	13.25	0.83	9.24	(2.7,8.9,5.1)	52
ONECR	-18.26	24.44	-10.08	30.62	-26.08	28.38	(4.1,10.1,1.5)	2688
TWO	-2.68	8.57	5.91	12.91	0.77	9.33	(2.7,9.2,4.9)	52
TWOCR	-22.83	22.10	-18.60	33.97	-20.91	24.12	(0.4,9.2,0.7)	1984
THREE	-2.08	8.60	5.98	11.98	0.74	8.48	(3.2,8.9,5.3)	46
THRCR	-27.13	26.72	-22.28	28.77	-17.36	37.60	(2.5,4.7,7.5)	1925
FOUR	-2.06	8.40	3.85	12.53	0.85	8.13	(2.7,8.9,4.8)	53
FOURCR	-25.85	35.01	-13.05	38.67	-22.94	30.18	(1.8,12.2,5.4)	3565
FIVE	-2.24	8.43	3.82	12.55	0.66	8.38	(2.5,9.0,4.8)	56
FIVECR	-20.53	20.69	-19.69	43.14	-33.51	33.67	(0.7,10.9,3.2)	3509
SIX	-1.11	7.92	3.67	13.69	0.37	8.81	(3.3,8.4,4.8)	47
SIXCR	-12.48	12.73	-8.69	26.06	-8.71	15.04	(1.0,9.3,2.5)	742
1) CI*	-3.5	9.0	4.0	14.0	-1.0	11.0		
CI + 6 A	-9.5	15.0	-2.0	20.0	-7.0	17.0		

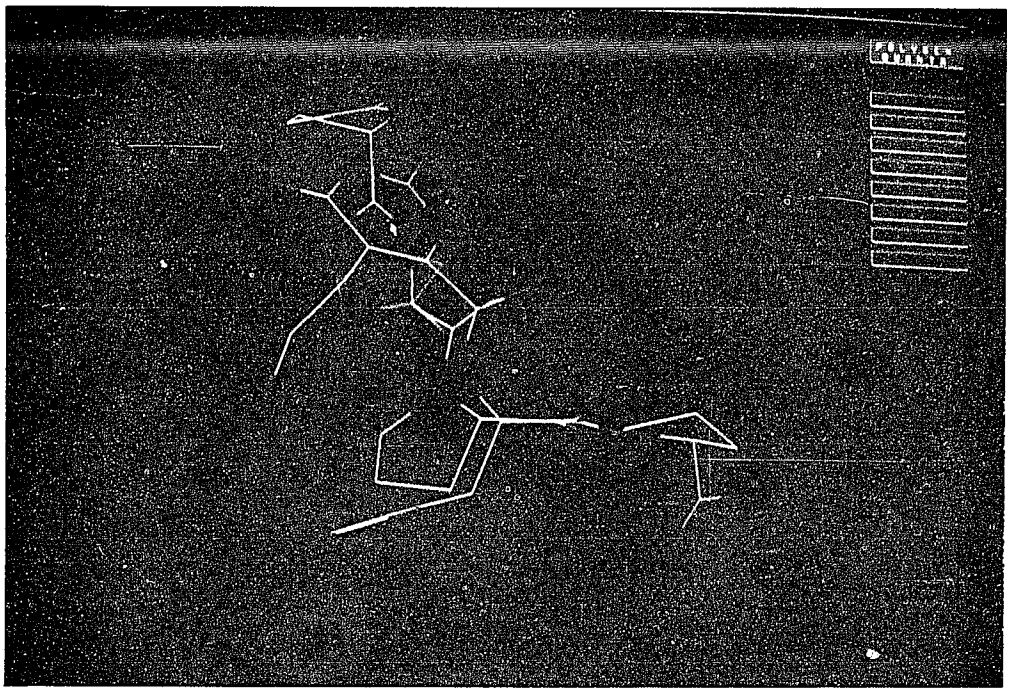
CI* = the consensus interval for all of the molecules.

The results from the computer program, SUMMABAT3, which compares the MEPs within the crystal contacts (CC) of the central molecule with the analogous MEPs of another molecule produced slight or no changes for the comparisons of the competitive inhibitor molecules. However, for the noncompetitive inhibitor molecules, there were found significantly better fits of the MEPs within the CCs by using this second superpositioning method. The results from the CC superpositioning method suggest that there may be alternative orientations for the noncompetitive molecules.

The results from the two superpositioning techniques were compared for the noncompetitive molecules. Analog V was superpositioned onto analog III by each of the two superpositioning methods. The RMS superpositioning method orientated the orotyl ring of analog V in a plane which is at right angles to the plane of the oxothiazolidine ring of analog III. The orientations derived from the RMS superpositioning criterion did not give as good a fit of the calculated MEPs. The second superpositioning technique produced several orientations for the orotyl ring which could fit the MEPs within the CCs of analog III better than did the first superpositioning technique. This suggested that analog V has at least one orientation which fits the orientation for analog III with a smaller absolute difference of the MEPs within the crystal contacts when compared to the RMS superpositioning method.

Figure 23 shows that the two molecules, analog V and analog III, can orient with their carbonyl oxygens aligned. However because there are more carbonyl oxygens in the orotyl ring of analog V, the aligned carbonyl oxygens are shifted down the lengths of molecules.

FIGURE 23



The reorientation of analog V is used in order to recalculate the MEP through the plane of the scissile peptide bond in order to see if there is an improved fit of the MEP with the MEP from analog III.

FIGURE 24

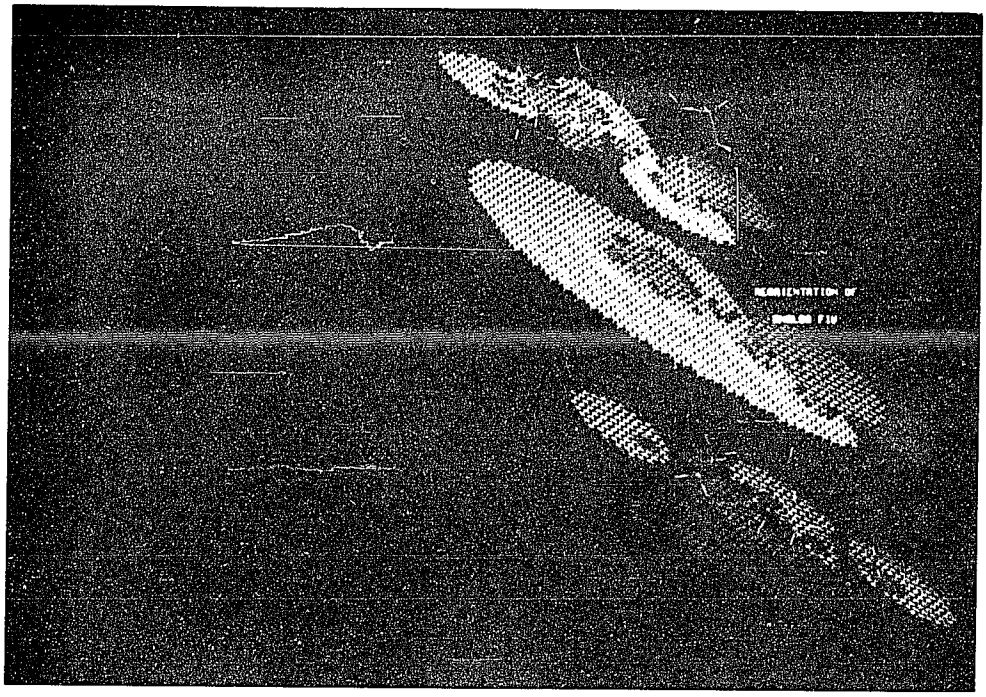


Figure 24 displays analog III with its associated MEP through the plane of the scissile bond and the two orientations of analog V with their associated MEPs. The third molecule in the bottom right corner is analog V from the first superpositioning method. Comparing analog V from the bottom right corner with analog III which appears at the top of the picture, it is relatively easy to see that the negative regions of the MEP which appear yellow, white and red are reversed with the positive violet and blue regions for the region around the prolineamide residue of the molecules. However, there is a better match between the positive and negative regions of the MEPs after the reorientation of analog V

which uses the second superpositioning criterion. This is especially noticeable in the region of the prolineamide ring where the positive and negative regions are reversed. The second superpositioning technique gives qualitatively better agreement between the MEP within the plane of the scissile bond for the comparison of analog III and analog V.

4) the placement of alternative orientations for the inhibitors within the environment of carboxypeptidase-A: Because of the similarities among the zinc containing enzymes (Argos et al., 1978 and Vallee & Auld, 1990), carboxypeptidase A is chosen as a representative metallo-protease in order to model the relative orientations of the molecules to the active site metal ion. The TRH-analog molecule III was superpositioned onto the slowly hydrolyzed substrate, glycyl-L-tyrosine (Gly-Tyr), within the active site of carboxypeptidase A. The relative orientations of the other TRH-analog molecules were positioned within the active site of carboxypeptidase A with respect to the position of the analog III molecule. Beginning with the first, RMS, superpositioning technique, the analyses suggest that the p-Glu ring region of the competitive inhibitors can protrude into a sterically hindered region of the enzyme.

When analog II is repositioned by accounting for the steric constraints imposed apon the five membered, pGlu ring, the zinc-oxygen distance becomes larger by about 1.00 Å for those competitive inhibitors with six-membered rings. The larger distance may be a major factor in explaining why these inhibitors are not metabolized by

PPII. For example, the zinc-oxygen distance for Gly-Tyr and analog III is 2.78 Å, whereas this distance is 3.82 Å for analog II which is a competitive inhibitor of PPII.

When the repositioned analog II is subsequently used as the template for the superpositioning of analog V, one of the carbonyl oxygens of the orotyl ring is 2.07 Angstroms from the zinc atom. This suggests that it may be possible for the carbonyl oxygen of the orotyl ring to reorient close to the zinc atom within the active site of the enzyme. A picture of this orientation is shown in Figure 25.

FIGURE 25

The measured oxygen-zinc distance is 2.07 Å, which is in good agreement with accepted distances (Argos et al., 1978; Kim & Lipscomb, 1990). The new position suggests that analog V can orient so that an alternative interaction may be made with the metal ion of the active site which may prevent the hydrolysis of the peptide bond. This may be due to the interaction of the orotyl ring carbonyl oxygen atom with the zinc rather than the peptide bond carbonyl oxygen as for the substrate molecules (analog III and TRH).

DISCUSSION:

Some of the accomplishments of this thesis are: 1) the successful purification of the enzyme, PPII, and the experimental determinations of the stabilities and apparent affinitties of the ten molecules with the enzyme, 2) the utilization of calculated physicochemical data from the crystal environments to understand the observed reactivities of the molecules, 3) the development of an alternative superpositioning program which superimposes the physicochemical data from the molecular crystal environments 4) the developement of computer programs which made the analyses of the large amount of data possible and 5) the identification of sites within the inhibitor molecules for possible modifications in order to create alternative inhibitors for PPII.

Analogs of TRH were examined by experiment and computer modeling in order to understand the molecular basis for their observed experimental reactivities with PPII. The experimental data is in good

agreement with recent data from Elmore, O'Connor and O'Cuinn (Elmore et al., 1990). They reported the K_is for four of the molecules which were tested with the enzyme, pyroglutamyl peptidase II (PPII). Their results are displayed for comparison with the data from Lanzara, Liebman and Wilk (Lanzara et al., 1989) in Table V below. It can be seen that most of the results from Elmore et al. are within two standard deviations of the values for the K_is from Lanzara et al.

Table V: Comparisons of the experimental values from Lanzara et al. with those of Elmore et al.

molecule:	K_is (Elmore et al.) μM	K_is (Lanzara et al.) μM
TRH	50	55 ± 8
CG3703	116	166 ± 46
Phe-TRH	125	187 ± 23
MK771	44	87 ± 26

From the experimental testing of the analogs of TRH with PPII three distinct groups of inhibitors are found. The first group is composed of the competitive inhibitors of PP II which are not cleaved by the enzyme. These are the analogs: I, II, and Phe-TRH. The second group is composed of the substrates of PPII which are cleaved by the enzyme. These are the molecules TRH and analog III. The third and last group is composed of the noncompetitive inhibitors of PPII. These are the analogs IV, V, and VI.

All of the analogs tested, except Phe-TRH (VII) and MK-771, have His-Pro-NH2 for the terminal residues and substituted rings for the pyroglutamyl ring of TRH. Since the K_i of Phe-TRH is nearly equal to the K_i of TRH, it would appear that the change from a charged imidazole to a neutral phenyl ring, does not appreciably change the binding affinity to the enzyme. This implies that the binding affinity is not sensitive to the charge of the molecule. This is supported by the broad pH range for PPII (Wilk & Wilk, 1989). However, the replacement of the imadazole with the phenyl ring prevents the cleavage of the molecule. This suggests some role for the histidyl group in hydrolysis. One possible explanation is that within the active site of the enzyme, the histidyl ring may hydrogen bond with an essential residue of the enzyme or the carbonyl oxygen of the C-terminal residue. The intramolecular hydrogen bonding may form a better leaving group and facilitate the cleavage of the peptide bond within the molecule. MK-771 has a 2-oxopiperidine ring substituted for the pyroglutamate ring of TRH and a thiazolidine ring substituted for the proline ring. It may be that the six membered oxopiperidine ring rather than the thiazolidine ring is what prevents the cleavage of MK-771 by PPII.

Although Phe-TRH has a similar configuration to TRH and binds relatively well to PPII, the x-ray crystal structure of the Phe-TRH analog has its phenyl ring shifted toward the amide nitrogen of the prolineamide which is consistent with the aromatic ring acting as a hydrogen bond acceptor (Levitt and Perutz, 1988). The interaction of the phenyl ring and the amide of the prolineamide is somewhat similar

to the intramolecular hydrogen bonding found for TRH in that the amide regions are drawn towards the second ring regions of the molecules. The major difference is that the pi electron cloud of the phenyl ring of Phe-TRH interacts with the amide-hydrogen, whereas the hydrogen of the Nε of the histidyl ring in TRH hydrogen bonds with the carbonyl oxygen of the amide. This confers a different electronic distribution on the Phe-TRH when compared to TRH itself (see the MEPs, Figure 19) and is consistent with the experimental observation that Phe-TRH is not cleaved by PPII.

The requirement of the histidyl residue for metabolism suggests that there may be at least two factors responsible for the nonmetabolism of Phe-TRH. First is that the difference of the electronic and steric structure of the phenyl versus the histidyl ring may make the His-Pro-NH2 a better leaving group than Phe-Pro-NH2. Second, the relative orientation of the phenyl ring may prevent an essential interaction with an essential residue within the active site. The experimental observations that substrates of PPII should be tripeptides or short tetrapeptides may depend on the ability of the carboxy terminal residues to be good leaving groups, or have rings in the second position which can weaken the scissile peptide bond when bound with a residue in the active site.

The substrate specificity of PPII requires that the substrate be a tripeptide (or a tetrapeptide with Gly as the last residue) with a five-membered ring in the first position followed by a histidyl residue in the second position. This requirement for a five-membered ring suggests

that there is a steric hindrance in the active site of the enzyme. For those competitive inhibitors which are not metabolized, the steric hindrance from their six-membered rings may prevent the carbonyl oxygen of the peptide bond from approaching close enough to interact with the metal atom of the enzyme. This requirement was associated with the cleavage of TRH and analog III by PPII from the PDM analysis and from placing the analogs within the environment of carboxypeptidase-A.

For the comparisons of the molecules which were competitive and cleaved with those which were not cleaved, both the RMS and CC superpositioning methods gave essentially identical results. From the subsequent analyses of these two experimental classes, it was found that there were regions which could be identified with the reactivities of each class. One of these regions was near the pyroglutamyl ring. The region was occupied by those analogs which have six-membered rings and were competitive inhibitors but not cleaved (analogs I and II) suggesting that a steric hindrance could occur within this region.

Also there were three other regions which strictly constrained the orientation of the histidyl residue. This suggests that of the three residues the second residue may be the most highly constrained residue. In addition, the carbonyl oxygen region of the histidyl residue was occupied by the cleaved molecules but not by the uncleaved molecules. This suggests that there may be an essential role for this oxygen within the active site perhaps analogous to the postulated binding between the carboxylate oxygen of the Tyr residue of the Gly-

Tyr substrate with the guanidinium nitrogen from the arginine-145 residue of carboxypeptidase A (Christianson & Lipscomb, 1986).

Adjacent to the beta carbon of the proline ring was another region which was occupied by those molecules which were competitive but not cleaved but was not occupied by either TRH or analog III. Taken together, these analyses suggest that the specificity of PPII may result from the strict orientations of each one of the residues within the active site.

All of the noncompetitive inhibitors (NCIs) in the third group have an sp2 configuration at the alpha carbon of the pGlu ring. The association of the sp2 geometry of the alpha carbon with noncompetitive enzyme kinetics suggests an important role for the geometry around the alpha carbon. Although it may be that the planar rings or another aspect of the geometry are what are important rather than the sp2 geometry around the alpha carbon. This is discussed in more detail below.

After the RMS superpositioning method, the pGlu substituent rings of the noncompetitive inhibitor (NCI) molecules were oriented 90° relative to the pyroglutamyl ring of TRH. This suggests that these molecules may have different orientations than those for the competitive molecules. This observation supports the classical formulation for noncompetitive inhibition by suggesting that the NCIs may be binding to a second, separate site on the enzyme. The second site may accept the more planar ring conformations for the N-terminal ring of the NCIs without allowing the ring conformations of the rings of

the substrates and competitive inhibitors. Although it seems unlikely that the smaller and more flexible rings of the competitive inhibitors and substrate molecules would not be able to bind to this second, putative site, it may be that the NCIs bind to the enzyme-substrate complex. From the perspective of the modelling done for this thesis, it seems difficult to see how two similar molecules could fit into the active site.

The CC superpositioning method found new orientations for the noncompetitive molecules. These new orientations were found to superimpose the calculated physicochemical data with a smaller difference than did the RMS superpositioning method. The new orientations demonstrate that the oxygen atoms of the molecules can assume a more complete alignment when the CC superpositioning method is used rather than the RMS method. The physical basis for this may be that the contribution of the oxygen atoms to the MEP is large and predominates within the regions of the crystal contacts. This would be a reasonable assumption based on Eckle and Stezowski's hydrogen bonding environment (Eckle E. and Stezowski J. (1985)). However, Eckle and Stezowski used a superpositioning technique which minimized the rms distance between the selected atoms of the molecules. This is similar to the first technique and would fail to fit possible reorientations of the molecules to the hydrogen bonding environment, since it is dependent on the method of selecting the atoms to be paired for the RMS distance measurement. There are at least two reasons why the RMS superpositioning method may not be adequate to make the

Although the CC superpositioning and the carboxypeptidase-A methods supported the conclusions from the first two computer analyses (PDM and RMS superpositioning) for the substrates and the competitive inhibitors, there was disagreement among the four computational methods for the analyses of the competitive and noncompetitive inhibitors. Various approaches are often necessary when examining these complex systems, but often more questions are raised than are solved. One question raised here is with the classical description for noncompetitive inhibition. The classical model for noncompetitive inhibition assumes that reversibility requires that there be a second site. However, the classical model rests on the assumptions that either there is a second binding site which can somehow prevent the enzyme from functioning or that the inhibitor binds to the enzyme-substrate complex. An inherent problem with the classical formulation is that there exists a second site which is independent of the influence from the competitive inhibitors and substrates, but can nonetheless influence the active site of the enzyme. This is usually assumed because a solution for the analytical equations requires the concept of a second, separate site. From the molecular-modeling perspective, it is difficult to understand how a second molecule might affect the enzyme-substrate complex without the binding of that molecule being affected in turn by the substrate binding. From the argument of microscopic reversibility the classical formulation seems hard to justify.

From the alternative orientations which were found to be possible for the NCIs within the constraints of the analyses, some interesting

possibilities may be entertained. The first possibility is that the NCIs may bind at a second site on the enzyme. This would be consistent with the classical scheme for noncompetitive enzyme inhibition and with the demonstrated reversibility of the reaction of the NCIs with PPII (see Results, Figure.12). The second possibility is that the NCIs bind with a different orientation to the active site in a manner which appears to be reversible. It may be that the NCI inhibitor molecules may have a low probability of making a moderately strong interaction which is nonetheless reversible. This is not modeled by the classical scheme for noncompetitive enzyme inhibition, but is consistent with some other experimental data (Rees and Lipscomb, 1981; Auld and Holmquist, 1974; Leff and Martin, 1986 and Connolly and Trayer, 1979) and may explain how pGluHis is a noncompetitive inhibitor of PPII (O'Connor and O'Cuinn, 1985; Elmore et al., 1990) without invoking a second binding site on the enzyme.

Rees and Lipscomb reported that the binding of indole acetic acid (IAA), a noncompetitive inhibitor, was to the same site in carboxypeptidase-A as for the competitive inhibitor Gly-Tyr. They refered to the report by Auld and Holmquist that IAA as well as two other inhibitors were both competitive and noncompetitive inhibitors of carboxypeptidase-A for ester and peptide hydrolysis, respectively. These inhibitors all had K_i's which were of equal magnitude for the competitive and noncompetitive inhibition for the same inhibitor molecule. These K_i's were in the range from 120 to 330 μM which suggests that these inhibitors are not behaving like a classical

irreversible inhibitor. It is interesting that from this work with inhibitors of PPII, the inhibitors which gave noncompetitive inhibition kinetics had K_i's ranging from 143 to 437 μM and were also shown to be reversible. Since Rees and Lipscomb obtained direct crystallographic evidence that there was only one site for the competitive and noncompetitive inhibitors of carboxypeptidase-A, this possibility should also be entertained for PPII.

O'Connor and O'Cuinn tested many structurally similar compounds with PPII and discovered that pGlu-Val, pGlu-Ala, pGlu-Asn-Gly and pGlu-His-Gly were all competitive inhibitors, whereas pGlu-His was a noncompetitive inhibitor with a K_i of 4.5 mM (O'Connor and O'Cuinn, 1985). Recently, Elmore et al. (Elmore et al., 1990) reported that of the fifteen peptides which they examined all except pGlu-His were competitive inhibitors of PPII. However, for the compounds tested by O'Connor and O'Cuinn, pGlu-Val and pGlu-Ala were competitive inhibitors while pGlu-His was a noncompetitive inhibitor. The K_i for pGlu-His is very high if the compound is binding at the active site in an irreversible manner. This implies the existence of a second site, by the classical model for noncompetitive inhibition. However, the structural similarities of pGlu-His with the other competitive inhibitors and substrates suggests that there exists a paradox in that the elements of recognition for a second, separate site exist on a molecule with structural elements which are identical to the substrate and many of the competitive inhibitors. That these elements of recognition for a second site are selectively present in pGlu-His, but absent from pGlu-

His-Pro, pGlu-His-Trp, pGlu-Val, pGlu-Ala, and pGlu-His-Gly is difficult to reconcile unless the binding is to the active site.

CONCLUSIONS:

Conclusions which were made include: 1) The regions which appear essential for the cleavage of the molecules are the five membered ring in the pGlu region together with a strict orientation of the imadazolium ring together with the carbonyl oxygens within and on either side of the scissile peptide bond. 2) Alternative regions which may be modified to give a nonmetabolized competitive inhibitor are the β carbons of the His and Pro-NH2 residues as well as the amino terminus within the pGlu ring. 3) The replacement of the pGlu ring by an orotyl or triazine ring confers noncompetitive inhibition on the inhibitor. This may be explained either by an alternative binding to the zinc atom by an oxygen from the planar rings of the inhibitor, or by the binding to a second site on the enzyme.

In summary, PPII displays a broader specificity for binding than for cleavage. The set of common patterns for the substrate molecules was modeled and found to depend on each of the three residues. The requirement for a molecule to be a substrate may involve both the formation of a stable leaving group and the correct orientation of the oxygens from the N-terminal ring, first peptide bond and the second or histidyl peptide bond. These requirements reduce the number of possible structural motifs for substrates. These motifs were computated by the inclusion of identical physicochemical properties within each of the three experimental classes of the molecules. The identity elements from one class was then compared to the identity elements from

another experimental class. In this way, regions of identity and differences were compared between the experimental classes. There are regions (ie. near the proline ring) where it appears that the addition of an extra group or groups may interfer with the cleavage of the molecule. This is consistent with the experimental observations that the TRH analogs RX74355 and RX77368 are not degraded *in vivo* and may explain why they are protected from PPII.

Theoretically, with a more powerful computer and *ab initio* calculations the system could be accurately characterized, however, the subsequent analyses across the experimental classes of molecules would require a similar approach to the approach used here. The choice of comparisons to be made and the rules which are necessary for making these comparisons are non trivial problems that are crucial to our understanding of differing experimental reactivities which are based apon molecular models. This work is an attempt to look at some of these comparisons and to use the available physicochemical data in order to understand and draw reasonable conclusions about the different experimental classes of reactivities which are observed with these structurally similar molecules. The hope is that this work will assist in solving these problems in the future.

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