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City University of New York PH.D. 1986

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BIOCHEMICAL STUDIES ON THE DNA BINDING FUNCTION OF THE CYCLIC AMP RECEPTOR PROTEIN OF *ESCHERICHIA COLI*

by

JESUS A. ANGULO

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

1986

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Jesus Angulo

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

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Abstract

BIOCHEMICAL STUDIES ON THE DNA BINDING FUNCTION OF THE CYCLIC AMP RECEPTOR PROTEIN OF *ESCHERICHIA COLI*

by

JESUS A. ANGULO

Adviser: Dr. Joseph S. Krakow

The cAMP receptor protein (CRP) is an allosteric protein in which binding of cAMP effects a conformational change with a consequent increased affinity for DNA. Binding of double-stranded deoxyribopolynucleotides and calf thymus DNA by cAMP-CRP confers protection against attack by trypsin, subtilisin, *Staph. aureus* V8 protease and clostripain. Of the single-stranded deoxy- and ribopolynucleotides tested, only $r(I)_n$ and $r(A)_n$ gave significant protection against attack by these proteases. Since the cutting sites for trypsin (Lys 130) and subtilisin (Leu 116) are not part of the C-terminal DNA binding domain, it would appear that binding of DNA may confer

conformational changes on other regions of cAMP-CRP.

In the absence of cAMP, CRP is resistant to proteolysis. Incubation of CRP-DNA with trypsin results in the accumulation of two novel fragments. CRP-DNA is partially sensitive to digestion by chymotrypsin but resistant to attack by subtilisin, the _Staph_. _aureus_ V8 protease and clostripain. Cleavage of CRP-DNA to fragments is accompanied by the loss of ^{3}H-cAMP binding activity. All double-stranded deoxyribopolynucleotides tested confer a conformation on CRP which can be readily attacked by trypsin. Single-stranded deoxy- and ribopolynucleotides have the same effect as double-stranded DNA's, with the exception of poly $d(A)_n$, poly $d(I)_n$ and poly $r(C)_n$ which do not bind or confer a conformation on CRP which is poorly attacked by trypsin. The 10,000 dalton fragment produced by trypsin digestion of the CRP-DNA complex has an N-terminal sequence identical to that of native CRP and terminates at Lys 89. The 6,000 fragment extends from Val 131 up to Arg 185.

Modification of the arginines with phenylglyoxal or butanedione results in loss of DNA binding activity. cAMP-CRP incorporates more ^{14}C-phenylglyoxal than unliganded CRP. Titration of the arginines with ^{14}C-phenylglyoxal to where

over 90% of the DNA binding activity is lost results in incorporation of one mole of reagent per mole of subunit. Kinetic analysis suggests that incorporation of one molecule of butanedione per molecule of CRP is suffucient to inactivate $^{3}H\text{-}d(A\text{-}T)_n$ binding activity. Modification by phenylglyoxal or butanedione does not affect cAMP binding activity. CRP modified with butanedione is rendered sensitive to chymotryptic attack in the absence of cAMP.

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TABLE OF CONTENTS

Copyright	ii
Approval	iii
Abstract	iv
Acknowledgements	vii
Table of Contents	ix
List of Tables	xi
List of Figures	xii
Abbreviations	xiv
Introduction	1
Materials and Methods	10
1. Source of materials	10
2. Growth of bacteria	10
3. Assay of ^{3}H-cAMP binding to CRP	11
4. Protein determinations	12
5. Purification of CRP	12
6. Polyacrylamide gel electrophoresis	12
7. Preparation of polynucleotides	13
8. Polynucleotide protection assay conditions	13
9. Preparation of ^{3}H-d(A-T)$_n$	14
10. Assay of ^{3}H-d(A-T)$_n$ binding to CRP	14
11. Conditions for the cleavage of the CRP-DNA complex	15
12. Elution of peptides from NaDodSO$_4$ polyacrylamide gels	15

13. Instrumentation for HPLC 16

14. Preparation of standard amino acid DABTH derivatives 16

15. Purification of solvents for micro-sequence analysis 17

16. N-terminal micro-sequence analysis of peptides 18

17. HPLC separation of DABTH-aa derivatives 19

18. Preparation of ^{14}C-phenylglyoxal from ^{14}C-acetophenone 20

19. Chemical modification of CRP with ^{14}C-phenylglyoxal or butanedione 21

Results 23

1. Effect of deoxyribopolymers and ribopolymers on the sensitivity of CRP to proteolytic attack 23

2. Effect on the conformation of CRP induced by deoxyribopolymers and ribopolymers 26

3. Chemical modification of CRP 31

Discussion 36

Tables 45

Figures 50

Bibliography 97

LIST OF TABLES

1. Effect of polynucleotides on digestion of cAMP-CRP by proteases. 45

2. Effect of single and double stranded polynucleotides on ^{3}H-cAMP binding by CRP digested with trypsin. 46

3. Molecular weight and N-terminal sequence of CRP fragments. 47

4. cAMP binding activity in CRP modified with butanedione and phenylglyoxal. 48

LIST OF FIGURES

1. Effect of cAMP and $d(A\text{-}T)_n$ on the rate of digestion of CRP by Staph. aureus V8 protease. 50

2A. Effect of cAMP and $r(I)_n$ on digestion of CRP by proteases. 52

2B. Effect of cAMP and $d(I)_n$ on digestion of CRP by proteases. 54

3. Effect of cAMP and $d(A\text{-}T)_n$ or $r(I)_n$ on digestion of CRP by trypsin. 56

4. Effect of cAMP and $d(A\text{-}T)_n$ on the rate of digestion of CRP by clostripain. 58

5. Effect of cAMP and heparin on digestion of CRP by proteases. 60

6. Digestion of CRP-DNA complex with proteases. 62

7. Time course of digestion of CRP-DNA complex with trypsin. 64

8. Loss of cAMP binding activity after trypsin digestion of CRP in the presence of DNA. 66

9. Effect of ionic strength on the trypsin sensitivity of the CRP-DNA complex. 68

10. Digestion of CRP-polymer complex with trypsin. 70

11. Purification of 10,000 and 6,000 dalton fragments of CRP. 72

12. Separation of a mixture of DABTH-amino acids on a Zorbax CDS column. 74

13. Gradient profile used in the separation of DABTH-amino acid derivatives. 76

14. Time course of digestion of CRP-heparin complex with trypsin. 78

15. ^{3}H-d(A-T)$_n$ binding activity of CRP modified with phenylglyoxal and butanedione. 80

16. Incorporation of ^{14}C-phenylglyoxal into CRP as a function of reagent concentration. 82

17. Time course of incorporation of ^{14}C-phenylglyoxal into CRP. 84

18. Inactivation of ^{3}H-d(A-T)$_n$ binding activity of CRP with butanedione. 86

19. Reaction order of CRP inactivation with respect to butanedione concentration. 88

20. Chymotrypsin digestion of CRP modified with butanedione (20 minutes). 90

21. Chymotrypsin digestion of CRP modified with butanedione (40 minutes). 92

22. Chemical structure of butanedione-arginine and phenylglyoxal-arginine. 94

23. Secondary structure of the CRP monomer. 96

Abbreviations used are: CRP, cyclic AMP receptor protein; cAMP, 3',5'-cyclic AMP; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetra-acetic acid; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; Tris, Tris-(hydroxymethyl) aminomethane; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; DABITC, 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate; PITC, phenylisothiocyanate; HPLC, high performance liquid chromatography; DABTH, 4-NN-dimethylaminoazobenzene 4'-thiohydantoin; PG, phenylglyoxal; BD, butanedione.

INTRODUCTION

The regulation of transcriptional activity is one of the predominant mechanisms accounting for the differential expression of genes in both prokaryotes and eukaryotes. In *E. coli*, the effects of cAMP are mediated by the cAMP receptor protein (CRP, also referred to as the catabolite gene activator protein or CAP). CRP is an allosteric DNA binding protein which mediates activation of transcription at various operons. This dissertation presents a series of experiments demonstrating the effect on conformation of CRP elicited by the binding of polydeoxyribonucleotides and polyribonucleotides. The effect of chemical modification of arginyl side chains on DNA binding activity and conformation of CRP is also documented.

Genetic analysis of the *E. coli* genome identified two loci involved in the modulation of the cAMP effect. Mutations at the *cya* and *crp* loci impair cAMP-dependent synthesis of the enzyme beta-galactosidase. Organisms with mutations at the *cya* locus show decreased levels of adenylate cyclase activity and can be induced for galactosidase synthesis by the addition of cAMP (Perlman and Pastan,

1969). The *crp*⁻ organisms cannot be induced for galactosidase with exogenously added cAMP, suggesting that the *crp* locus codes for a protein factor which acts as a receptor for the cyclic nucleotide (Perlman et al., 1970). Screening of fractions from a cell lysate for cAMP binding activity led to the purification of a protein factor, CRP, which mediated the induction of galactosidase synthesis (Zubay et al., 1970). Purified CRP was shown to direct *lac* mRNA synthesis *in vitro* in the presence of *lac* DNA, cAMP, RNA polymerase and nucleoside triphosphates (deCrombrugghe et al., 1971).

Studies *in vitro* with DNA fragments containing the *lac* promoter sequence have been used to probe the site of interaction of cAMP-CRP with DNA. DNA sequencing and DNAse I or exonuclease III protection experiments have placed the site of interaction of cAMP-CRP in the *lac* promoter between residues -72 and -47 relative to the transcription initiation site (Simpson, 1980). The protected sequence contains an inverted repeat centered around residues -61/-62. Guanines protected by cAMP-CRP from attack by dimethylsulfate are placed symmetrically around the dyad. Since one

dimer of CRP binds to this region, it is postulated that when cAMP-CRP is bound to this sequence the two symmetrically arranged elements must sense approximately the same protein configuration (Crothers and Fried, 1982). A second cAMP-CRP binding site, P2, has been discovered in the lac promoter at +1 to +24 from the transcription start site (Schmitz, 1981). This sequence does not exhibit the dyad of symmetry present in the primary site. The affinity of binding was found to be 20-30 fold lower than the primary site (Crothers and Fried, 1982).

Transcription activation at the lac promoter by cAMP-CRP has been shown to be affected by the conformation of the DNA. Experiments utilizing inhibitors of DNA gyrase have shown a relationship between the inhibition of this enzyme and the immediate and specific inhibition of expression of the wild-type lac promoter (Sanzey, 1979). Activation by cAMP-CRP has been approached at the level of the reactions catalyzed by RNA polymerase during initiation. Transcription initiation has been postulated to involve the interaction of the RNA polymerase holoenzyme with the promoter

to form the inactive closed complex. In the second step, the closed complex isomerizes to form the transcriptionally active open complex (Chamberlin, 1974). Kinetic analysis by McClure and coworkers using the steady-state rate of synthesis of the abortive initiation product ,ApApUpU, has shown that cAMP-CRP increases the rate of formation of the closed transcriptionally inactive complex without affecting the rate constant for the isomerization step (Malan et al., 1984). They concluded that cAMP-CRP acts directly to enhance the initial binding of RNA polymerase to the promoter and to position the enzyme at the proper start site.

Sequence analysis of promoters activated by CRP has revealed important features about the CRP binding site. Digestion of cAMP-CRP-DNA with DNAse I yields a fragment approximately 25 bp long. The bases protected from chemical attack and the mutations that prevent CRP binding are located within the 25 bp fragment. The fragment contains a highly conserved consensus sequence, 5'TGTGA3', which is always located distally in the DNA fragment. CRP has higher affinity for promoters (<u>lac</u> promoter) where the sequence 6 bp downstream of the TGTGA

motif generates a twofold symmetry. In cases where no corresponding symmetrical element is generated (*gal* promoter), the sequence 6 bp downstream of the TGTGA motif is still essential for CRP activity *in vitro* and *in vivo*. It is postulated that the second subunit of the dimer interacts with this sequence (Ebright, 1982). It is of interest to note that no uniformity has been observed between the distance from the transcription start point and the CRP binding site.

Binding of cAMP by CRP causes an increase in the binding constant for specific sequences (sites on promoters which are activated by CRP). The data suggest that one molecule of cAMP bound to the CRP dimer suffices for stable binding of the complex to specific DNA sites (Garner and Revzin, 1982). Interaction of the cAMP-CRP complex with its target DNA induces a change in conformation in the DNA from the B to the C form as measured by circular dichroism (Saxe and Revzin, 1979). CRP can also bind to DNA non-specifically in the absence of cAMP; the binding is highly cooperative. It has been postulated that in the absence of ligand, binding of the first molecule of CRP facilitates the binding of subsequent

molecules through protein-protein interactions until the DNA molecule is coated by CRP (Takahashi et al., 1979). Clusters of CRP with pBR322 DNA molecules have been observed by electron microscopy (Chang et al., 1981).

Evidence for a change in protein conformation induced by cAMP came from limited proteolysis experiments. Unliganded CRP was shown to be resistant to trypsin and chymotrypsin, among other proteases tested, as judged by SDS-polyacrylamide gel analysis. Binding of cAMP resulted in the production of an amino terminal core fragment which retained cAMP binding activity but lacked DNA binding activity (Krakow and Pastan, 1973; Eilen, Pampeno and Krakow, 1978). CRP undergoes DTNB-mediated intersubunit disulfide crosslinking in the presence of cAMP, indicative of a change in conformation in liganded CRP which causes two available sulfhydryls to come to proximity (Eilen and Krakow, 1977). Chemical crosslinking with o-phenylenedimaleimide (o-PDM) is faster in the presence of cAMP. CRP modified with o-PDM is resistant to proteolysis in the absence of cAMP. Liganded o-PDM-CRP digested with chymotrypsin produces

discrete peptide fragments (Pampeno and Krakow, 1979). Physical evidence for a ligand-induced change in the conformation of CRP came from small-angle X-ray scattering studies which showed a reduction in the radius of gyration of CRP induced by the ligand (Kumar, Murthy and Krakow, 1980). In 0.5% SDS, chymotrypsin cleaves CRP into a fragment 9.5K and a fragment of 13K. The 9.5K is amino-terminal and associates mostly in dimers, suggesting that this part of the protein contains sites involved in subunit interaction. The 13K fragment is C-terminal and retains poly $d(A\text{-}T)_n$ binding activity in the presence or absence of cAMP (Aiba and Krakow, 1981).

The crystal structure of cAMP-CRP was solved at 2.9A by McKay and Steitz (1981). The crystal structure showed the presence of two structural domains in the complex, a larger amino-terminal domain (residue 1-135) and a smaller carboxy-terminal domain (residue 136-204). The two domains are separated by a cleft. The large domain is composed of a short alpha-helix followed by eight beta-sheets folded into a beta-roll and two additional alpha-helices (B and C). A cAMP molecule is buried in the interior of each subunit of the

dimer with residues from both subunits making contact with the ligand. The 6-amino group of the adenine ring of cAMP interacts with thr 127 on one subunit and ser 128 on the other (McKay et al., 1982). Most of the subunit contact sites are provided by alpha-helix C, which is 24 residues long. The carboxy-terminal domain consists mostly of three alpha-helices connected by short beta-sheet structures. One salient feature of the smaller domain is the arrangement of alpha-helices E and F. The two F helices of the dimer are related to one another by a two-fold axis of symmetry. In addition, the distance between their main axis is about 34A and the planes of their main axis run approximately parallel to each other (McKay and Steitz, 1981). Amino acid side chains in the F helices are postulated to interact with bases in two successive major grooves of left-handed B DNA. The E helices are believed to interact with phosphate groups on the DNA backbone (McKay and Steitz, 1981). The crystal structure of cAMP-CRP has provided a model for the structure of the protein; however, it must be borne in mind that the protein may assume other conformations in solution.

The research comprised in this dissertation was undertaken to explore the effect of DNA binding on the conformation of the cAMP-CRP complex in solution and to elucidate the role of arginyl side chains in DNA binding. The first two-thirds present evidence for an effect on the conformation of CRP when bound to DNA in the presence and absence of cAMP. The chemical modification experiments presented in the last third of this thesis implicate an essential arginine in maintaining the conformation of the C-terminal domain of CRP

MATERIALS AND METHODS

1. Source of Materials

Materials were obtained as follows: coomassie brilliant blue R-250 from Research Organics Inc.; sodium dodecyl sulfate, TEMED and bis-acrylamide from Bio Rad; acrylamide from Serva; calf thymus DNA, bovine serum albumin, sperm whale myoglobin, bovine pancreas trypsin inhibitor, poly $r(I)_n$, poly $r(U)_n$, poly $r(A)_n$, poly $r(C)_n$, poly $d(I)_n$, poly $d(T)_n$, poly $d(A)_n$, poly $d(C)_n$, trypsin, chymotrypsin, subtilisin, clostripain, butanedione and phenylglyoxal from Sigma; $d(A\text{-}T)_n$, poly $d(T)_n$:poly $d(A)_n$, $d(I\text{-}C)_n$, $d(A\text{-}U)_n$, $d(A\text{-}BrU)_n$ and the Klenow fragment of DNA polymerase I from P-L Biochemicals; *Staph. aureus* V8 protease from Miles; ^{3}H-cAMP and ^{3}H-TTP from ICN; butyl acetate, pyridine, heptane and ethyl acetate from J. T. Baker; HPLC grade water and acetonitrile from Burdick and Jackson Laboratories Inc.; sequanal grade PITC, DABITC, trifluoroacetic acid and heparin-agarose from Pierce; ^{14}C-acetophenone and Aquasol II from New England Nuclear.

2. Growth of Bacteria

E. coli KLF41/JC1553 pHA7, which is an over-

producing strain carrying the CRP gene in a 910 bp insert of pBR322, was the generous gift of Hiroji Aiba of Kyoto University, Kyoto, Japan. The cells were grown at 37°C in media of the following composition (per liter): 10 gm of yeast extract, 5.6 gm of KH_2PO_4, 28.9 gm of K_2HPO_4, 10 mg of thiamine-HCl, 10 gm of glucose and 50 mg of ampicillin. The cells were harvested at mid log and the pellet frozen at -20°C.

3. Assay of ^{3}H-cAMP Binding to CRP

The method used is that described by Pastan et al. (1974). The ^{3}H-cAMP binding assay mix consisted of the following (total volume 5 mL): 4 mg/mL casein (as carrier protein), 20mM 5'AMP, 20mM potassium phosphate buffer, pH 7.7, 2uM cAMP and 50 uL of ^{3}H-cAMP (27Ci/mmol). The specific activity of the ^{3}H-cAMP mix was 3,000 cpm/pmol. The binding reaction was carried out in a polypropylene tube (10X75 mm) in 50 uL of mix, 2-4 ug of CRP and 50 uL of H_2O to give a final volume of 100 uL. After 5 minutes on ice, 0.5 mL of cold saturated ammonium sulfate, pH 8.0, was added and the tube allowed to stand on ice for another 5 minutes. The tube was centrifuged at 10,000 rpm for 10 minutes and the precipitate dissolved in 0.3 mL

of 0.1N NaOH. The sample was counted in 5 mL of Aquasol II. The control tube contained the mix minus CRP.

4. Protein Determinations

Protein content was determined by the method of Schaffner and Weissmann (1973) using bovine serum albumin as standard.

5. Purification of CRP

CRP was purified to homogeneity as described by Eilen, Pampeno and Krakow (1978), except that the final step involved chromatography on a 2X12 cm heparin-agarose column. The protein was eluted with a linear gradient (500 mL) of NaCl from 0.05M to 0.6M. The fractions (8 mL each) were assayed for ^{3}H-cAMP binding activity. The active fractions were found to be homogeneous by polyacrylamide gel electrophoresis in $NaDodSO_4$. CRP was stored at -20°C in 10mM potassium phosphate buffer, pH 7.0, 1.0mM EDTA, 0.1mM DTT and 0.2M NaCl at a protein concentration of 10mg/mL.

6. Polyacrylamide Gel Electrophoresis

Polyacrylamide gels in $NaDodSO_4$ were prepared as described by Laemmli (1970). Electrophoresis

was performed in a temperature controlled (15°C) double slab electrophoresis apparatus from Bio Rad at 30 mA per slab for 2 hours. The gels were stained for 2 hours at room temperature in 0.2% coomassie brilliant blue R-250, 50% methanol and 10% acetic acid. The gels were destained overnight in a diffusion destainer against destaining solution (water:acetic acid:isopropyl alcohol, 8:1:1 v/v).

7. Preparation of Polynucleotides

Polynucleotides were dissolved in 10mM Tris buffer, pH 8.0, 0.5M NaCl and 1.0mM EDTA at a concentration of 8.0 A_{260}/mL and dialyzed for 24 hours at 4°C against 2 liters of buffer, then for another 24 hours against the same buffer solution but without NaCl. They were stored at -20°C. The single stranded polynucleotides were of molecular weight greater than 100,000 as specified by the manufacturer.

8. Polynucleotide Protection Assay Conditions

The reaction mixture contained 20 ug of CRP, 24 ug of DNA or RNA, 10mM Hepes buffer, pH 8.0, 0.1mM DTT, 1.0mM EDTA and 10mM NaCl at a final volume of 100 uL. The mixture was

digested for the indicated time at 37°C and terminated by the addition of 5 uL of 20mM PMSF (in 50% v/v DMSO). To this was added 5 uL of mercaptoethanol and 10 uL of electrophoresis sample buffer (0.5M Tris buffer, pH 8.7, 50% glycerol and 10% $NaDodSO_4$). After 5 minutes at 95°C, 30 uL were layered on a 15% polyacrylamide gel with a 4.75% stacking gel.

9. Preparation of $^3H\text{-}d(A\text{-}T)_n$

$^3H\text{-}d(A\text{-}T)_n$ was synthesized as described by Jovin et al. (1969) using the Klenow fragment of DNA polymerase I. The reaction mixture was made 1.0M in NaCl and 10mM in EDTA to stop the reaction. The mixture was immediately filtered through a nitrocellulose membrane filter for deproteinization and the filtrate dialyzed first against 0.5M NaCl, 1.0mM EDTA, 10mM Tris buffer, pH 8.0, and then against the same buffer solution but at 0.1M NaCl. Dialysis was performed at 4°C overnight against 2 liters of buffer solution. The tritiated copolymer was stored at -20°C in the second dialysis buffer.

10. Assay of $^3H\text{-}d(A\text{-}T)_n$ Binding to CRP

Varying amounts of CRP (1-20 ug) were incu-

bated for 5 minutes at 37°C in 50mM Tris buffer, pH 8.0, 20mM KCl, 0.1mM cAMP and 9.0 nmol of ^{3}H-d(A-T)$_n$ (specific activity of 3,000 cpm/nmol) in a final volume of 100 uL. After addition of 0.3 mL of 50mM KCl, the reaction mixture was filtered through a nitrocellulose membrane filter which had been soaked for 30 minutes in 0.1N KOH and then stored in 50mM Tris buffer, pH 8.0, and 1.0mM EDTA. After drying, the filters were counted in 5 mL of toluene-based liquifluor.

11. Conditions for the Cleavage of the CRP-DNA Complex

The reaction mixture contained (final volume 100 uL): 20 ug of CRP, 12 ug of calf thymus DNA (a 5.0 A_{260}/mL solution dialyzed overnight at 4°C against 10mM Tris buffer, pH 8.0, and 1.0mM EDTA), 10mM Hepes buffer, pH 8.0, 0.1mM DTT, 1.0mM EDTA and 20mM NaCl. The mixture was digested with trypsin (1% w/w) for 30 minutes at 37°C and terminated by the addition of 10 uL of 20mM PMSF. To this was added 5 uL of mercaptoethanol and 10 uL of electrophoresis sample buffer. 30 uL were layered on the gel.

12. Elution of Peptides from $NaDodSO_4$ Polyacry-

lamide Gels

The procedure used was essentially that of Hager and Burgess (1980). The protein bands were visualized in cold 0.25M KCl, cut out from the gel with a clean razor blade and washed 3 times (10 minutes each) with deionized water at room temperature. The gel was gently homogenized (by hand) and 1.5 mL of elution buffer (50mM Tris buffer, pH 8.0 and 0.2% $NaDodSO_4$) added. After incubation overnight at 4°C (or 3 hours at 37°C), the tubes were spun in a clinical centrifuge to pellet the gel fragments and the aqueous layer removed with a Pasteur pipet. The protein solution was dialyzed overnight at 4°C against 0.5M acetic acid, lyophilized and used for amino terminal analysis.

13. Instrumentation for HPLC

High performance liquid chromatography was performed on a Gilson HPLC system. Constant temperature was achieved by circulating water through an aluminum jacket connected to a Brinkmann Instruments incubator.

14. Preparation of Standard Amino Acid DABTH Derivatives

Amino acid DABTH derivatives were prepared essentially as described by Chang (1981). 0.5 mg of amino acid was dissolved in 100 uL of triethylamine/acetic acid buffer (50 mL of water + 50 mL of acetone + 0.5 mL of triethylamine + 5 mL of 0.2M acetic acid, pH 10.65) and treated with 50 uL of DABITC solution (4 nmol/uL in acetone). The solution was flushed with nitrogen for 10 seconds and heated at 53°C for 1 hour (in a 1 mL Eppendorf tube). The solvent was evaporated (at room temperature) with a stream of nitrogen. The residue was dissolved in 50% (v/v) trifluoroacetic acid (in water) and heated at 53°C for 50 minutes. The acid was evaporated with a stream of nitrogen. The DABTH derivative was dissolved in 0.5 mL of 70% (v/v) ethanol and used as standard sample. Amino acid DABTH derivatives were analyzed on a Zorbax ODS column from DuPont. Sample sizes of 5-20 uL were usually injected.

15. Purification of Solvents for Micro-sequence Analysis

Pyridine was purified as described by Chang et al. (1978). It was distilled over KOH (10 gm/L) first, then over ninhydrin (1 gm/L) and then over KOH again (10 gm/L). Pyridine was stored protected

from light at -20°C. Heptane (400 mL) was treated with 6 grams of activated charcoal and filtered through a Whatman 3MM fluted filter. The filtrate was distilled over ninhydrin (1 gm/L). The same purification procedure was employed in the purification of ethyl acetate and butyl acetate. The solvents were stored in the dark at room temperature. Trifluoroacetic acid (200 mL) was distilled over 3 grams of $CaSO_4$ and stored dry in a dark place.

16. N-terminal Micro-sequence Analysis of Peptides

N-terminal sequence analysis was performed as described by Chang (1981). Peptides (4-6 nmol) were dissolved in 80 uL of aqueous 50% (v/v) pyridine in a 1 mL reacti-vial (Pierce) equipped with a screw cap. The peptide was treated with 40 uL of DABITC solution (10 nmol/uL in pyridine). The tube was flushed with N_2 for ten seconds, sealed and placed in a heating block at 53°C for 50 minutes. After the first coupling, 10 uL of pure PITC was added and the second coupling allowed to proceed for 30 minutes at 53°C. The excess reagents and by-products were extracted four times with 450 uL portions of heptane:ethyl acetate (2:1, v/v). The aqueous phase was dried

over N_2 and the residue dissolved in 100 uL of trifluoroacetic acid. Cleavage was allowed to proceed for 15 minutes at 53°C. The sample was dried over N_2 and the cleavage product dissolved in 100 uL of H_2O. The cleavage product was extracted with 300 uL of butyl acetate. After this, the aqueous phase was dried and used for the second cycle. The butyl acetate was evaporated over N_2 and the residue dissolved in 100 uL of aqueous 50% trifluoroacetic acid (v/v). Conversion took place at 53°C for 45 minutes. The aqueous acid was evaporated and the thiohydantoin derivative dissolved in 100 uL of aqueous 45% acetonitrile (v/v). Samples of 10-20 uL were analyzed on a Zorbax ODS column.

17. HPLC Separation of DABTH-aa derivatives

DABTH-aa derivatives were chromatographed on a Zorbax ODS column (DuPont) at 22°C. Solvent A was 35mM sodium acetate buffer, pH 5.0, and solvent B acetonitrile. The gradient was as follows: 45% to 70% solvent B from 0-20 minutes; 70% solvent B from 20-30 minutes; 80% acetonitrile from 30-40 minutes; and 45% solvent B from 40-50 minutes. The flow rate was 1 mL/min and the chart speed at 0.1mm/sec. The detector sensitivity was set at 0.02 AUFS at 420 nm wavelength.

18. Preparation of ^{14}C-phenylglyoxal from ^{14}C-acetophenone

^{14}C-phenylglyoxal was prepared by the method of Riley and Gray (1947) scaled down to the uL range. The reaction was carried out in a 1 mL glass reacti-vial (Pierce) equipped with a screw cap. The following solution was prepared: 0.4 gm of SeO_2 was partially dissolved in 3 mL of freshly distilled dioxane. To this was added 110 uL of H_2O and the mixture heated to 55°C. When the SeO_2 was completely dissolved, 130 uL were transferred to the reaction vial and the reaction started by the addition of 4 uL of ^{14}C-7-acetophenone (2.65 mCi/mmol). The tube was tightly sealed and the contents heated to 80°C in a heating block for 4 hours (with occassional stirring). When the tube cooled to room temperature, the contents were applied as a streak 2 cm from the bottom of a 20X20 cm silica gel plate (Analtech). The plate was developed with petroleum ether:ethyl acetate (1:1, v/v). The band corresponding to ^{14}C-phenylglyoxal (R_f = 0.8) was visualized under a UV lamp and the silica scraped with a spatula. The ^{14}C-phenylglyoxal was eluted from the silica with 1 mL of H_2O and filtered through a nitrocellulose membrane filter. The solution was stored in the

refrigerator protected from light. The concentration of phenylglyoxal in solution was determined by the extinction coefficient (E_{253} = 12,600 at pH 7.0) given by Kohlbrenner and Cross (1978).

19. Chemical Modification of CRP with ^{14}C-phenylglyoxal or butanedione

CRP (40 ug) was allowed to react with ^{14}C-phenylglyoxal (5,000 cpm/nmol) at a reagent concentration of 1.0mM in 50mM sodium borate buffer, pH 8.0, and 0.1mM cAMP (when present) at 37°C. The reaction was carried out in a glass tube at a final volume of 100 uL. The modification reaction was terminated by the addition of arginine at a 6-fold molar excess and dialyzed overnight at 4°C against 2 liters of 50mM sodium borate buffer, pH 8.0, 20mM NaCl and 1.0mM EDTA. 4 ug of protein were taken into 400 uL of 5% TCA and filtered through a nitrocellulose membrane filter. The wet filter was allowed to stand for 2 hours in 5 mL of Aquasol II and counted for one minute. The same procedure was used for modification with butanedione, except that the reagent concentration was 2.5mM. Since butanedione is not available in

radioactive form, the extent of modification was measured as a function of loss of ^{3}H-d(A-T)$_n$ binding activity.

RESULTS

1. Effect of Deoxyribopolymers and Ribopolymers on the Sensitivity of CRP to Proteolytic Attack

CRP is resistant to the *Staph. aureus* V8 protease in the absence of cAMP; addition of cAMP leads to the production of a resistant amino-terminal core. Figure 1 shows that addition of $d(A-T)_n$ to cAMP-CRP protects CRP in the complex from hydrolysis. In another experiment it was shown that $d(A-T)_n$ does not inhibit the *Staph. aureus* V8 protease (data not shown). The $d(A-T)_n$ was present at saturating levels of 112 mol base pairs per mol CRP dimer. Garner and Revzin (1981) have determined that the CRP molecule interacts with 13 bases in DNA.

The same degree of protection against the *Staph. aureus* V8 protease was afforded CRP by $d(I-C)_n$, $d(A-U)_n$, $d(A-BrU)_n$ and calf thymus DNA. These double-stranded polymers protected cAMP-CRP against attack by trypsin and subtilisin but not against chymotryptic digestion (data not shown). The CRP core peptide produced after subtilisin digestion had an N-terminal sequence identical to that of the native pro-

tein up to leucine 116 (Tsugita et al., 1982), indicating a molecular weight of 12,800. Based on mobility after $NaDodSO_4$-polyacrylamide gel electrophoresis, the estimated molecular weight of the CRP cores produced by chymotrypsin and Staph. aureus V8 protease were 15,000 and 18,800 respectively. Digestion of cAMP-CRP with trypsin yielded two major fragments of molecular weights 14,300 and 18,500. Continued incubation with trypsin resulted in a gradual loss of the larger fragment. The generation of two dissimilar fragments by trypsin may be a consequence of a partial symmetry of the two carboxyl-terminal domains in the CRP dimer (McKay and Steitz, 1981).

Figures 2A and B show the effect of $r(I)_n$ and $d(I)_n$ on digestion of CRP by the four proteases used. Both $r(I)_n$ and $d(I)_n$ protected against Staph. aureus V8 protease, with little effect seen in protecting against chymotrypsin hydrolysis. Of the two polymers, $r(I)_n$ afforded somewhat more protection against subtilisin and trypsin, indicative of higher affinity for this single-stranded ribopolymer or different CRP conformations with varying sensitivities to proteolysis. It is worth noting that, in the presence of $d(I)_n$, where no protection against

trypsin was observed (Fig. 2B), the cAMP-CRP was digested more readily with no detectable intermediate of molecular weight 18,500.

We have assayed eight single-stranded polynucleotides for their effect on digestion of cAMP-CRP by subtilisin, trypsin, Staph. aureus V8 protease and chymotrypsin. The results are summarized in Table I. All single-stranded polynucleotides protected against the Staph. aureus V8 protease, while none protected against chymotrypsin. Only $r(I)_n$ and, to a lesser extent, $r(A)_n$ protected against subtilisin and trypsin. $r(C)_n$ showed about 50% protection against subtilisin. With regard to trypsin, the results in Table I indicate that cAMP-CRP has greater affinity for single-stranded purine ribopolymers or that these purine ribopolymers induce a conformation in CRP which protects against the protease. The affinity of cAMP-CRP for $r(I)_n$ was not affected by a change in ionic strength between 10 and 50 mM NaCl. As seen in Figure 3, the degree of protection afforded by $r(I)_n$ paralleled that by $d(A-T)_n$.

In CRP, 9 out of the 11 arginine residues are located in the carboxyl proximal domain (Aiba et al., 1982). Figure 4 shows that interaction

of cAMP-CRP with $d(A-T)_n$ protects some arginine(s) against hydrolysis by clostripain, an arginine-specific protease. This is indicative of a change in conformation around this region or steric blockage by DNA. The molecular weight of the clostripain core was approximately 15,600, indicating that hydrolysis occurs at arginine 142.

Heparin is a sulfonated polysaccharide with a high negative charge density. The data in Figure 5 show that the interaction of heparin with cAMP-CRP must be different from the interaction with single- or double-stranded polynucleotides. Heparin afforded protection against chymotrypsin, whereas no single- or double-stranded polynucleotide did. Heparin did not inhibit the activity of any of the proteases used in this study (data not shown).

2. Effect on the Conformation of CRP Induced by Deoxyribopolymers and ribopolymers

CRP is relatively resistant to attack by proteolytic enzymes such as trypsin, chymotrypsin, subtilisin, clostripain and the Staph. aureus V8 protease (Angulo and Krakow, 1984). The cAMP-CRP complex is rapidly attacked by these enzymes to produce N-terminal cores which retain cAMP

binding activity but no longer bind DNA (Krakow and Pastan, 1973). As shown in Figure 6, the DNA-CRP complex formed at low ionic strength was rendered sensitive to hydrolysis by trypsin and partially to chymotrypsin. The Staph. aureus V8 protease, subtilisin and clostripain (data not shown) did not attack the DNA-CRP complex more rapidly than the rate at which they act on unliganded CRP. The results suggested that binding of DNA by CRP altered its conformation in such a way that regions of the protein otherwise inaccessible in unliganded CRP, cAMP-CRP or cAMP-CRP-DNA were made available to attack by trypsin and chymotrypsin. Prolonged incubation of DNA-CRP with chymotrypsin resulted in accumulation of a 4,000 Da fragment (data not shown). Digestion of DNA-CRP by trypsin resulted in the formation of fragments which are smaller than those observed after the action of this protease on the cAMP-CRP complex. Two fragments were formed following incubation of DNA-CRP with trypsin: 10,000 and 6,000 daltons respectively. The core polypeptides formed after digestion of cAMP-CRP with trypsin were 18,500 and 14,300 daltons.

A comparison of the rate of digestion and the nature of the fragments formed during the incubation of cAMP-CRP and DNA-CRP with trypsin is shown in Figure 7. The relative rate of formation of the polypeptide fragments indicated that the cAMP-CRP complex was more rapidly attacked than the DNA-CRP complex. It was also apparent that the sites at which trypsin cuts CRP in each complex were different. Trypsin digestion of DNA-CRP directly resulted in the formation of the 10,000 and 6,000 dalton fragments without the detectable intermediation of the larger 18,500 and 14,300 tryptic fragments which were formed from the cleavage of cAMP-CRP.

Coincident with the fragmentation of DNA-CRP by trypsin was a loss of cAMP binding activity (Figure 8). Under the conditions of the reaction there was only a small loss of cAMP binding activity following incubation of unliganded CRP with trypsin. Loss of cAMP binding provides a convenient assay for determining the ability of polynucleotides to confer trypsin sensitivity to CRP (Table 2). The effect could be seen with a variety of synthetic polyribonucleotides and polydeoxyribonucleotides

in addition to the calf thymus DNA used previously. All of the double-stranded deoxypolymers sensitized CRP to attack by trypsin with the consequent loss of cAMP binding activity. $d(A)_n$, $d(I)_n$ and $r(C)_n$ were relatively ineffective whereas binding of $r(A)_n$, $r(I)_n$ and $d(C)_n$ by CRP resulted in the trypsin mediated loss of cAMP binding activity.

The fragmentation of DNA-CRP by trypsin is sensitive to ionic strength. As seen in Figure 9, raising the NaCl concentration from 30 to 90 mM resulted in retention of cAMP binding activity. Polyacrylamide gel electrophoresis in SDS showed that at 90mM NaCl CRP was not attacked by trypsin in the presence of DNA (data not shown). This is probably due to dissociation of the DNA from the protein at the higher salt concentration.

As seen in Figure 10, tryptic digestion of cAMP-CRP resulted in formation of the 18,500 and 14,300 fragments; the cAMP-CRP-DNA complex was relatively resistant to attack by trypsin. The $d(A-T)_n$-CRP complex was attacked to form the 10,000 and 6,000 dalton fragments. The CRP complexes formed with $r(A)_n$ or $r(I)_n$

in the presence of cAMP were relatively resistant to attack by trypsin; cAMP-CRP plus added $d(A)_n$ or $r(C)_n$ remained sensitive to trypsin. There is an apparent correlation between protection of cAMP-CRP and the digestion of CRP (in the absence of cAMP) with the polymer present during incubation with trypsin.

The fragments formed by cleavage of cAMP-CRP and DNA-CRP with trypsin were isolated by preparative $NaDodSO_4$ polyacrylamide gel electrophoresis (Figure 11). To establish the position of the fragments in CRP (Table 3) the N-terminal sequence of each fragment was determined by the DABITC-PITC double coupling method of Chang (1981). In Figure 12 is shown the separation of 16 DABTH-amino acids on a Zorbax ODS column using the gradient system shown in Figure 13. The 10,000 and 14,300 fragments produced by trypsin digestion are N-terminal in CRP. Based on the molecular weight of each fragment determined by the method of Weber and Osborn (1969) and the amino acid sequence of CRP (Aiba et al., 1982), it is suggested that trypsin cuts DNA-CRP at Arg87 or Lys89 to form the 10,000 dalton fragment. The failure of the arginine-specific protease, clostripain, to attack DNA-CRP

indicated that Lys89 may be the C-terminal residue of the 10,000 fragment. The 14,300 fragment was apparently formed by cutting CRP at Lys130 with trypsin. The 6,000 fragment was derived from the C-terminal DNA binding domain of CRP (McKay and Steitz, 1981). Its N-terminal sequence begins with Val131. Based on the molecular weight of the fragment, it is estimated that the C-terminal is at Arg.185.

As seen in Figure 14, the CRP-heparin complex is cleaved by trypsin to fragments of 10,000 and 6,000 daltons respectively. In the presence of heparin, a sulfonated polysaccharide with a high negative charge density, the 10,000 fragment is readily digested. The N-terminal sequence was not determined for these fragments.

3. Chemical Modification of CRP

Chemical modification of proteins has been a useful approach in the study of the relationship between structure and function. Since 9 of the 11 arginines in CRP are located in the C-terminal domain (McKay and Steitz, 1981), I have explored the role of arginine residues in the function of CRP. In this study, phenylglyoxal and butanedione have been used for their known

specificity toward arginine residues in proteins (Takahashi, 1968; Vallee and Riordan, 1968).

Figure 15 shows the DNA binding characteristics of CRP in the presence and absence of cAMP. In the absence of cAMP, CRP binds to DNA cooperatively. Binding of the first molecule of CRP facilitates binding of other molecules through protein-protein contacts until the DNA molecule is coated with CRP (Takahashi et al., 1979; Saxe and Revzin, 1979). CRP modified with 1.0mM phenylglyoxal or 2.0mM butanedione for 60 minutes is inactive in DNA binding activity in the presence or absence of cAMP.

As can be seen in Figure 16, incorporation of ^{14}C-phenylglyoxal into CRP at various reagent concentrations results in greater incorporation of the radioactive label in the absence of cAMP. This is indicative of greater accessibility of the arginines to the reagent in unliganded CRP, or greater reactivity of the arginines.

A time course of ^{14}C-phenylglyoxal incorporation into CRP (at 1.0mM reagent) shows that after 40 minutes of reaction, nearly one mole of reagent is incorporated per mole of subunit. As seen in Figure 17, in the pre-

sence of cAMP the reaction plateaus when a stoichiometry of 1:1 is reached, suggesting that in liganded CRP the other arginines are less reactive or less exposed than in unliganded CRP. In the sodium borate buffer system, a phenylglyoxal: arginine stoichiometry of 1:1 has been reported (Sandmeier and Christen, 1982). Addition of DNA to the reaction mixture does not afford protection to the putative critical arginine (data not shown). The phenylglyoxal incorporated into CRP is very stable since only 5-10% of the label dissociates after overnight dialysis in the sodium borate buffer system.

Since CRP is a cAMP binding protein, it had to be determined whether modification with phenylglyoxal or butanedione resulted in loss of ^{3}H-cAMP binding activity. As seen in Table 4, modification to the point where all ^{3}H-d(A-T)$_n$ binding activity was lost, and beyond, did not result in loss of ^{3}H-cAMP binding activity.

Modification of CRP with butanedione resulted in loss of ^{3}H-d(A-T)$_n$ binding activity. Since butanedione is not available in radioactive form, a kinetic approach was employed to determine the number of butanedione molecules needed to inactivate one CRP molecule. As seen

in Figure 18, inactivation of ^{3}H-d$(A-T)_n$ binding activity by butanedione follows pseudo-first order kinetics. The reaction can be represented by the following equation:

$$E + nI \xrightarrow{K''} EIn$$

where K" is the apparent second-order rate constant, E is the CRP, and n is the number of molecules of inhibitor I (butanedione). Since the inactivation follows pseudo-first order kinetics, the pseudo-first order rate constant $K' = K''(I)^n$. The logarithmic conversion of this equation, $\log K' = \log K'' + n\log (I)$ is an equation for a straight line. A plot of log K' vs. log (butanedione) should give a straight line with the slope equal to n, the number of butanedione molecules needed to inactivate one CRP molecule. Figure 19 shows a plot of the log K' vs. log (I). The straight line has a slope of 1.11, suggesting that modification of one arginine residue results in inactivation of ^{3}H-d$(A-T)_n$ binding activity in CRP.

CRP modified with butanedione is cleaved by chymotrypsin at a faster rate than native CRP. As seen in Figures 20 and 21, unliganded CRP-butanedione was attacked by chymotrypsin with resultant accumulation of unstable intermediates.

Chymotryptic attack of liganded CRP-butanedione resulted in accumulation of a low molecular weight peptide which is larger than the peptide derived from digestion of native cAMP-CRP. A control was run to show that the effects were not due to incomplete removal of the cAMP by overnight dialysis (data not shown).

DISCUSSION

The results show that binding of cAMP-CRP to double-stranded deoxypolymers and single-stranded deoxy- and ribopolymers can confer protection against cleavage by proteases. The degree of protection varies with regard to the protease used. The Staph. aureus V8 protease cleaves cAMP-CRP to produce a core peptide of molecular weight 18,800 by hydrolysis at glutamic acid 171. All of the polynucleotides tested protect cAMP-CRP against cleavage by the Staph. aureus V8 protease. The crystal structure of cAMP-CRP shows two consecutive alpha helices (E and F), which are postulated to interact with DNA (McKay and Steitz, 1981; McKay, Weber and Steitz, 1982). Glutamic acid 171 resides in the E helix and, since it has not been implicated in DNA contact, the protection effects seen may be due to conformational effects or steric blockage resulting from polymer binding. Since no large fragments are formed under these conditions, it is evident that binding of polynucleotides also protects CRP against attack by Staph. aureus V8 protease at glutamic acids 181 and 191.

All of the polynucleotides tested, whether double or single stranded, fail to protect cAMP-CRP from chymotryptic digestion. Chymotrypsin cuts at phenylalanine 136. This phenylalanine residue is present in the linker region between the N-terminal and C-terminal domains. This region in the cAMP-CRP is relatively unstructured and accessible to attack by chymotrypsin even in the ternary complex. In contrast, in unliganded CRP, this region is not attacked by chymotrypsin, suggesting a locally more compact conformation.

All the double-helical DNAs tested show protection of cAMP-CRP from attack by subtilisin and trypsin. Of the single-stranded polynucleotides tested only $r(I)_n$ and $r(A)_n$ show significant protection against these two proteases whose cleavage sites are far removed from the two-helix DNA binding motif. These results indicate that DNAs or single-stranded polynucleotides for which CRP has high affinity may induce a conformational change in the protein that extends through the large C helix, making the sensitive bonds inaccessible to the proteases. There are reports in the literature indi-

cating that the N-terminal CRP core produced by subtilisin digestion binds to a 298-bp fragment containing the lac promoter, and that the binding is cAMP-dependent (Clore, Gronenborn and Davies, 1983). Another report shows that the subtilisin core causes a conformational change in $d(A\text{-}T)_n$ in the presence of cAMP, suggesting some kind of interaction (Takahashi et al., 1982).

The data presented suggest that arginine 142 may be involved in either contact with the DNA or maintenance of conformation since clostripain fails to cut cAMP-CRP at arginine 142 in the presence of $d(A\text{-}T)_n$. The protection observed may also be due to steric blockage by the DNA.

The experiments presented suggest that DNA binding has a profound effect on the conformation of cAMP-CRP. The observed effects induced by DNA binding extend beyond the C-terminal domain of CRP.

In the absence of cAMP, CRP shows cooperative binding to DNA (Takahashi et al., 1979). Formation of the DNA-CRP complex induces a CRP conformation that is readily attacked by tryp-

sin and chymotrypsin. DNA-CRP remains as resistant to attack by clostripain, subtilisin and the _Staph. aureus_ V8 protease as unliganded CRP. CRP bound to DNA is partially digested by chymotrypsin, with only a fragment of 4,000 daltons accumulating. Incubation of DNA-CRP with trypsin results in the formation of two fragments and a concomitant loss of cAMP binding activity. CRP is degraded to form a N-terminal 10,000 dalton fragment and a 6,000 dalton C-proximal fragment. All of the double-stranded polydeoxyribonucleotides and several of the single-stranded polydeoxyribonucleotides and polyribonucleotides tested render CRP sensitive to cleavage by trypsin. CRP is slowly cleaved by trypsin in the presence of $d(A)_n$, $d(I)_n$ and $r(C)_n$ indicative of a weaker affinity of CRP for these polynucleotides or a conformational state induced in CRP by these polymers which renders the susceptible bonds less accessible to trypsin. In contrast to the results presented above, Garner and Revzin (1981) found that the interaction of CRP with $d(T)_n$ was weaker than with $d(A)_n$ at NaCl concentrations below 17.5mM.

It appears likely that the conformational

changes elicited in CRP on polynucleotide binding is largely a consequence of electrostatic interactions with amino acid side chains present in both the large and small domains of CRP. Takahashi et al. (1982) have shown that the N-terminal alpha CRP core (formed by digestion of cAMP-CRP by subtilisin) and CRP are able to bind $d(A\text{-}T)_n$ at low ionic strength in the absence of cAMP. It is thus possible that in the absence of cAMP, interaction with DNA occurs with both the large and small domains of CRP. That the nature of the binding in the absence of cAMP is largely electrostatic can be inferred from the observation that trypsin sensitivity in the presence of DNA is reversed by increasing the NaCl concentration above 30mM.

Tryptic digestion of DNA-CRP results in the loss of cAMP binding activity and the formation of the N-terminal and C-proximal fragments. The crystal structure of CRP indicates that cAMP interacts with amino acid side chains present in the beta pocket and alpha C helix of the large domain (McKay et al., 1982). The 10,000 dalton fragment probably terminates at Lys89 and will have lost the beta-8 strand as

well as all of the alpha C helix. It is possible that the beta-8 strand may be involved in maintaining the conformation of the cAMP binding domain. The alpha C helices may be involved in transmitting to the DNA binding domain the conformational signal generated in the large domain following binding of cAMP.

The C-proximal 6,000 dalton fragment extends from Val131 to Arg185 and contains the alpha E helix implicated in the electrostatic interaction of CRP with the phosphate backbone of DNA. This CRP fragment also contains part of the alpha F helix which protrudes from the surface of CRP to interact with bases present in the major groove of DNA (McKay and Steitz, 1981; McKay et al., 1982). The electrostatic interaction of lysine and arginine side chains in this region of CRP with DNA may sterically block access of this region to tryptic attack. It is of interest to note that whereas DNA-CRP is susceptible to cleavage by trypsin, the cAMP-CRP-DNA complex is resistant to tryptic attack. The cAMP-CRP complex is cleaved to form the N-terminal 14,300 dalton CRP core fragment. The region between Lys100 and Lys130 is available to tryptic attack in DNA-CRP and resistant in unliganded CRP,

cAMP-CRP and cAMP-CRP-DNA. The region from Arg185 to the C terminus of CRP is digested by trypsin in DNA-CRP and cAMP-CRP but remains resistant in both unliganded CRP and cAMP-CRP-DNA. It is obvious that CRP is able to effect a variety of conformational states not seen in the crystal structure of the cAMP-CRP complex.

CRP is an allosteric protein which undergoes a change in conformation upon cAMP binding (Krakow and Pastan, 1973). Liganded CRP binds specifically to promoter sequences upstream of the transcription initiation site in catabolite sensitive operons resulting in the activation of gene expression (de Crombrugghe et al., 1984). Modification of CRP with ^{14}C-phenylglyoxal at various reagent concentrations resulted in greater incorporation of the label into unliganded CRP. The data suggest that arginine residues in CRP are more reactive or accessible in unliganded CRP, indicative of different conformational states in the protein. A similar observation was made when the available cysteine residues in CRP were modified with DTNB (5-5'-dithiobis(2-nitrobenzoic acid)). Eilen and Krakow (1977) showed that when cAMP was present, the rate of formation of the TNB anion (2-nitro-

5-thiobenzoate) was significantly faster than when cAMP was omitted from the reaction mixture. These observations attest to the conformational flexibility of the CRP molecule in solution.

The time course of incorporation of ^{14}C-phenylglyoxal into cAMP-CRP suggests that there is one arginine residue which is readily titrated by the label (within 50 minutes), whereas the rest of the arginine residues react slowly over a prolonged period of time. In unliganded CRP, the arginine residues seem to be equally reactive up to the longest time point determined. One mole of radiolabel is incorporated per subunit of the CRP dimer. An alternative explanation is that 2 molecules of phenylglyoxal are needed to modify one arginine residue in one subunit of the dimer. There are ample examples in the literature where a phenylglyoxal:arginine stoichiometry of 2:1 has been observed (Takahashi, 1968).

Since butanedione is not commercially available in radioactive form, a kinetic analysis was employed to determine the number of molecules of butanedione needed to inhibit DNA binding activity in CRP (Hollenberg et al., 1971; Marcus

et al., 1976; Marschel and Bodley, 1979). The kinetic data showed that one molecule of inhibitor (butanedione) is sufficient to inactivate DNA binding activity in the CRP molecule. This suggests that two functional subunits are needed for stable binding to DNA.

CRP modified with butanedione is cleaved by chymotrypsin in the absence of cAMP, with concomitant accumulation of a peptide fragment identical in molecular weight to the N-terminal core peptide produced by chymotryptic digestion of cAMP-CRP (Eilen, Pampeno and Krakow, 1978). In BD-CRP, this putative core peptide is unstable and readily decomposes to fragments smaller in molecular weight. It is clear that modification of the essential arginine residue results in a conformational change in the DNA binding domain of the protein. It is suggested that the essential arginine residue plays a role in maintaining the conformation of the C-terminal domain of CRP.

POLYNUCLEOTIDES	SUBTILISIN	TRYPSIN	STAPH. AUREUS V8 PROTEASE	CHYMOTRYPSIN
d(T)n	-	-	+++	-
d(C)n	+	+	+++	-
r(U)n	+	-	+++	-
r(C)n	++	-	+++	-
d(A)n	-	-	+++	-
d(I)n	+	-	+++	-
r(I)n	+++	+++	+++	-
r(A)n	++	++	+++	-

TABLE I: Effect of polynucleotides on digestion of cAMP-CRP by proteases. All single stranded DNA's and RNA's were present at 13 ug per 20 ug CRP. In addition, the reaction mixture contained 10mM Hepes buffer, pH 8.0, 10mM NaCl, 1.0mM EDTA, 0.1mM DTT and 0.1mM cAMP (total volume 100 uL). The proteases were present at the following concentrations (w/w): subtilisin, 0.5%; trypsin, 1%; Staph. aureus V8 protease, 2.5%; and chymotrypsin, 1%. The mixture was incubated for 30 minutes at 37°C.

- = no protection

+ = partial protection

++ = about 50% protection

+++ = complete protection

POLYNUCLEOTIDE	PMOLES OF 3H-cAMP BOUND/3 ug CRP
none	3.53
poly d(A-T)n	0.18
poly d(I-C)n	0.10
poly d(A-U)n	0.05
poly d(A-BrU)n	0.25
poly d(A)n: poly d(T)n	0.25
calf thymus DNA	0.24
poly r(U)n	0.51
poly d(T)n	0.12
poly r(A)n	0.07
poly d(A)n	1.92
poly r(C)n	2.11
poly d(C)n	0.08
poly r(I)n	0.14
poly d(I)n	1.72

Table 2: Effect of single and double stranded polynucleotides on 3H-cAMP binding by CRP digested with trypsin. The reaction contained 13 ug of polynucleotide, 20 ug CRP, 10mM NaCl, 10mM Hepes buffer, pH 8.0, 1.0mM EDTA and 0.1mM DTT at a final volume of 100 uL. The mixture was digested with trypsin (1% w/w) for 30 minutes at 37°C. The reaction was stopped with 5 uL of 20mM PMSF and 15 uL assayed for 3H-cAMP binding activity.

POLYPEPTIDE	MOLECULAR WEIGHT	N-TERMINAL SEQUENCE
CRP	23,500	H_2N-VAL(1)-LEU-GLY-LYS-
Subtilisin core	12,800	H_2N-VAL(1)-LEU-GLY-LYS-
Trypsin core	14,300	H_2N-VAL(1)-LEU-GLY-LYS-
Large DNA fragment	10,000	H_2N-VAL(1)-LEU-GLY-LYS-
Small DNA fragment	6,000	H_2N-VAL(131)-GLY-ASN-LEU-

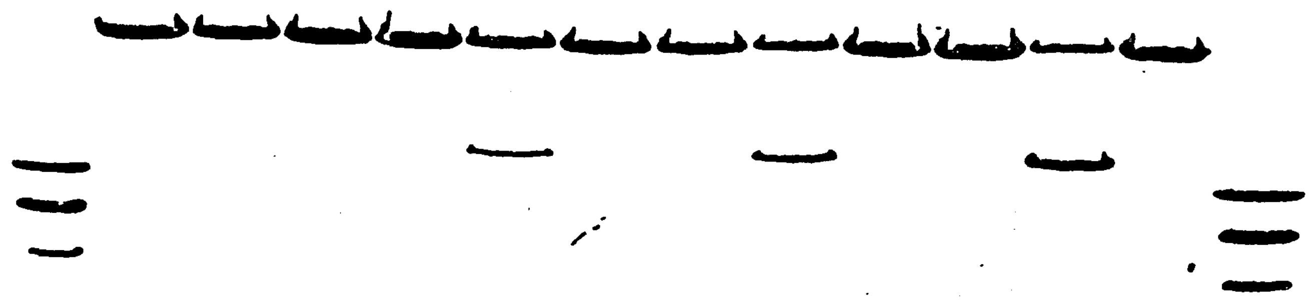
Table 3 : Molecular weight and N-terminal sequence of CRP fragments. Molecular weight determinations were based on mobility in SDS-polyacrylamide gel electrophoresis using the system of Weber and Osborn (1969). The N-terminal sequences were determined by the DABITC-PITC double coupling method of Chang (1981)

<u>cAMP BINDING ACTIVITY IN CRP MODIFIED WITH BUTANEDIONE AND PHENYLGLYOXAL</u>

modification time in minutes	pmoles of 3H-cAMP bound	
	butanedione	phenylglyoxal
0	2.40	2.40
20	2.65	2.03
40	2.75	2.05
80	2.43	1.86

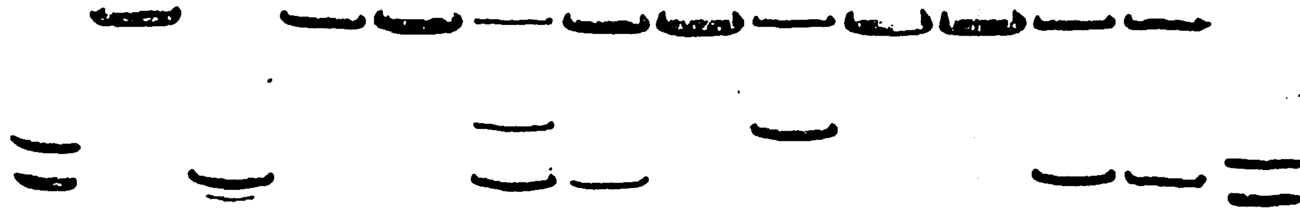
Table 4: At the time indicated, 5.6 ug of CRP were incubated for 5 minutes at $4^{o}C$ in 1.0uM 3H-cAMP (specific activity 6,000 cpm/pmol), 0.01M potassium phosphate buffer, pH 7.7, and 0.01M 5'AMP (final volume 100uL). After 5 minutes, 20 uL of 10mg/mL casein and 0.5 mL of saturated ammonium sulfate, pH 8.0, were added. The tube was allowed to stand at $4^{o}C$ for 10 minutes and then spun at 10,000 rpm for 10 minutes. The pellet was dissolved in 0.3 mL of 0.1N NaOH and counted for one minute in Aquasol II.

Figure 1: Effect of cAMP and $d(A-T)_n$ on the rate of digestion of CRP by the Staph. aureus V8 protease. CRP was digested for the time indicated at 2.5% protease (w/w). The reaction mixture consisted of 20 ug of CRP, 16 ug of $d(A-T)_n$, 50mM NaCl, 10mM Hepes buffer, pH 8.0, 0.1mM DTT, 1.0mM EDTA and 0.1mM cAMP where indicated. The products of digestion were resolved by SDS-polyacrylamide (15%) gel electrophoresis. The markers from top to bottom in lanes 1 and 14: sperm whale myoglobin, molecular weight 17,800; cytochrome c, 12,600; and bovine pancreas trypsin inhibitor, 6,500.



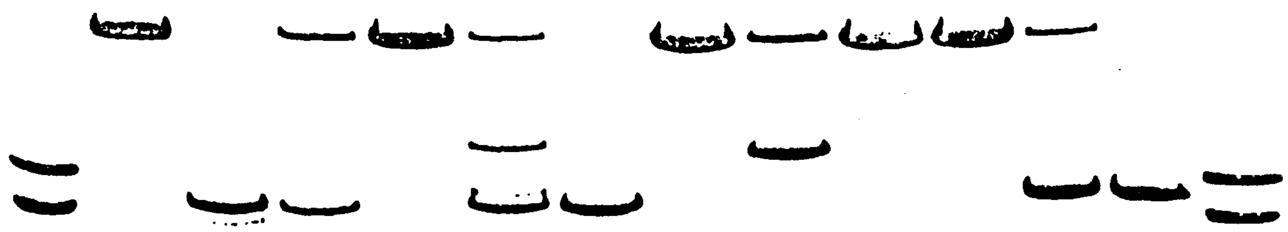
cAMP	−	+	+	−	+	+	−	+	+	−	+	+
$d(A-T)_n$	−	−	+	−	−	+	−	−	+	−	−	+
MINUTES		10			20			40			80	

Figure 2A: Effect of cAMP and r(I)n on digestion of CRP by proteases. Reaction conditions: 10mM NaCl, 10mM Hepes buffer, pH 8.0, 1.0mM EDTA, 0.1mM DTT, 0.1mM cAMP (where present), 20 ug of CRP and 13 ug of r(I)n. Total ionic strength = 20mM. Proteases were present at the following concentrations (w/w): subtilisin, 0.5%; trypsin, 1%; *Staph. aureus* V8 protease, 2.5%; and chymotrypsin, 1%. CRP was incubated with the proteases for 30 minutes at 37°C, total reaction volume 100 uL. Molecular weight markers in the first and last lanes: sperm whale myoglobin, 17,800 and cytochrome c, 12,600.



	SUB			TRY			SAP			CHY		
cAMP	−	+	+	−	+	+	−	+	+	−	+	+
$r(I)_n$	−	−	+	−	−	+	−	−	+	−	−	+

Figure 2B: Effect of cAMP and d(I)n on digestion of CRP by proteases. Reaction conditions and molecular weight markers are the same as in figure 2A above. 13 ug of d(I)n per 20 ug of CRP.



cAMP	−	+	+	−	+	+	−	+	+	−	+	+
$d(I)_n$	−	−	+	−	−	+	−	−	+	−	−	+
	SUB			TRY			SAP			CHY		

Figure 3: Effect of cAMP and d(A-T)n or r(I)n on digestion of CRP by trypsin. CRP was reacted with 1% trypsin (w/w) at 37°C for 30 minutes. The reactions contained 10mM Hepes buffer, pH 8.0, 0.1mM DTT, 1.0mM EDTA, 0.1mM cAMP (when present), 20 ug of CRP and 16 ug of d(A-T)n or 13 ug of r(I)n. NaCl as indicated in the figure. Molecular weight markers in lanes 1 and 14 are: sperm whale myoglobin, 17,800; cytochrome c, 12,600; and bovine pancreas trypsin inhibitor, 6,500.

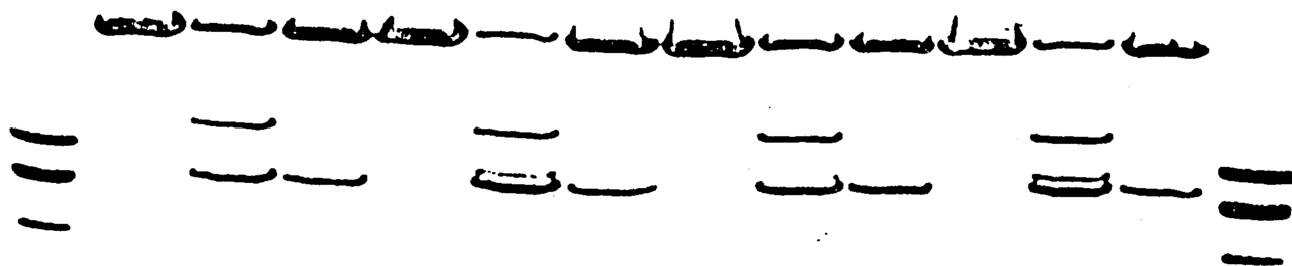
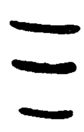
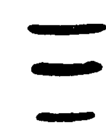

cAMP − + + − + + − + + − + + cAMP
d(A−T) − − + − − + − − + − − + $r(I)_n$
NaCl 50 mM 10 mM 50 mM 10 mM NaCl

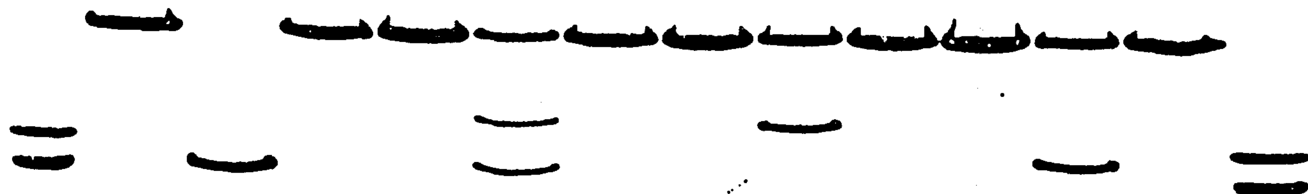
Figure 4: Effect of cAMP and d(A-T)n on the rate of digestion of CRP by clostripain. The reaction mixture contained 10mM Hepes buffer, pH 8.0, 10mM NaCl, 5mM $CaCl_2$, 20 ug of CRP and 16 ug of d(A-T)n. Clostripain was present at 2.5% (w/w). After 30 minutes at 37°C, the reaction was terminated by addition of $NaDodSO_4$ to a concentration of 1% followed by heating at 95°C for 5 minutes. Molecular weight markers in the first and last lanes are: sperm whale myoglobin, 17,800; cytochrome c, 12,600; and bovine pancreas trypsin inhibitor, 6,500.





cAMP	−	+	+	−	+	+	−	+	+	−	+	+
$d(A-T)_n$	−	−	+	−	−	+	−	−	+	−	−	+
MINUTES		10			20			40			80	

Figure 5: Effect of cAMP and heparin on digestion of CRP by proteases. The reaction (final volume 100 uL) contained 10mM Hepes buffer, pH 8.0, 10mM NaCl, 1.0mM EDTA, 0.1mM DTT, 0.1mM cAMP, 20 ug of CRP and 10 ug of heparin. Proteases were present as follows (w/w): subtilisin, 0.5%; trypsin, 1%; Staph. aureus V8 protease, 2.5%; and chymotrypsin, 1%. After 30 minutes at 37°C, the reactions were terminated by the addition of 5 uL of 0.02M PMSF followed by heating in 1% $NaDodSO_4$ at 95°C for 5 minutes. Molecular weight markers in lanes 1 and 14 are: sperm whale myoglobin, 17,800; cytochrome c, 12,600; and bovine pancreas trypsin inhibitor, 6,500.



	SUB			TRY			SAP			CHY		
cAMP	−	+	+	−	+	+	−	+	+	−	+	+
HEPARIN	−	−	+	−	−	+	−	−	+	−	−	+

Figure 6: digestion of CRP-DNA complex with proteases. The reaction contained (final volume 100 uL) 20 ug of CRP, 16 ug of calf thymus DNA, 10mM Hepes buffer, pH 8.0, 10mM NaCl, 1.0mM EDTA, 0.1mM DTT, and 0.1mM cAMP where indicated. The proteases were used at the following concentrations (w/w): subtilisin, 0.5%; trypsin, 1%; Staph. aureus V8 protease, 2.5%; and chymotrypsin, 1%. After 30 minutes at 37°C the reactions were terminated by the addition of 5 uL of 20mM PMSF. Molecular weight markers in lanes 1 and 14 are: sperm whale myoglobin, 17,800; cytochrome c, 12,600; and bovine pancreas trypsin inhibitor, 6,500.

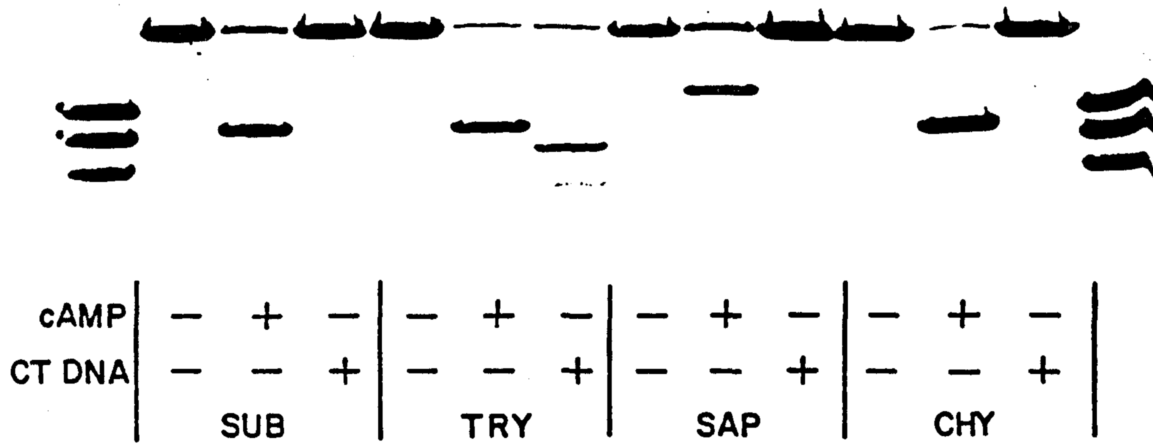

cAMP
− + − − + − − + − − + −
CT DNA
− − + − − + − − + − − +
SUB
TRY
SAP
CHY

Figure 7: time course of digestion of CRP-DNA complex with trypsin. The reaction consisted of 20 ug of CRP, 16 ug of calf thymus DNA, 10mM Hepes buffer, pH 8.0, 10mM NaCl, 1.0mM EDTA, 0.1mM cAMP (where present), 0.1mM DTT and trypsin at 1% (w/w). The mixture (total volume 100 uL) was reacted for 30 minutes at 37°C and terminated by the addition of 5 uL of 20mM PMSF. 30 uL were layered on the gel. Molecular weight markers in lanes 1 and 13 are: sperm whale myoglobin, 17,800; cytochrome c, 12,600; and bovine pancreas trypsin inhibitor, 6,500.

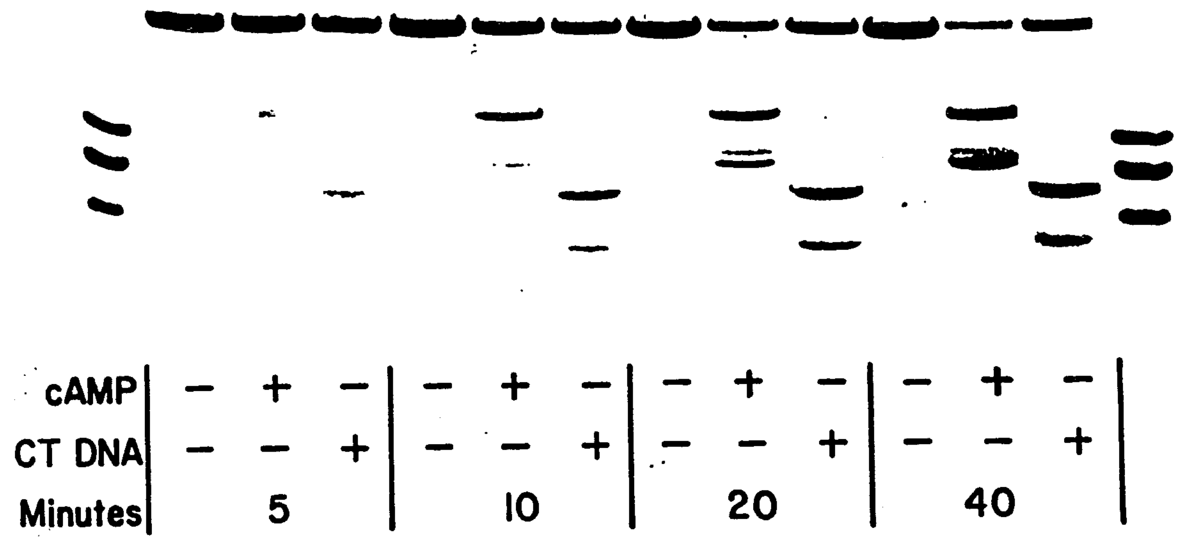
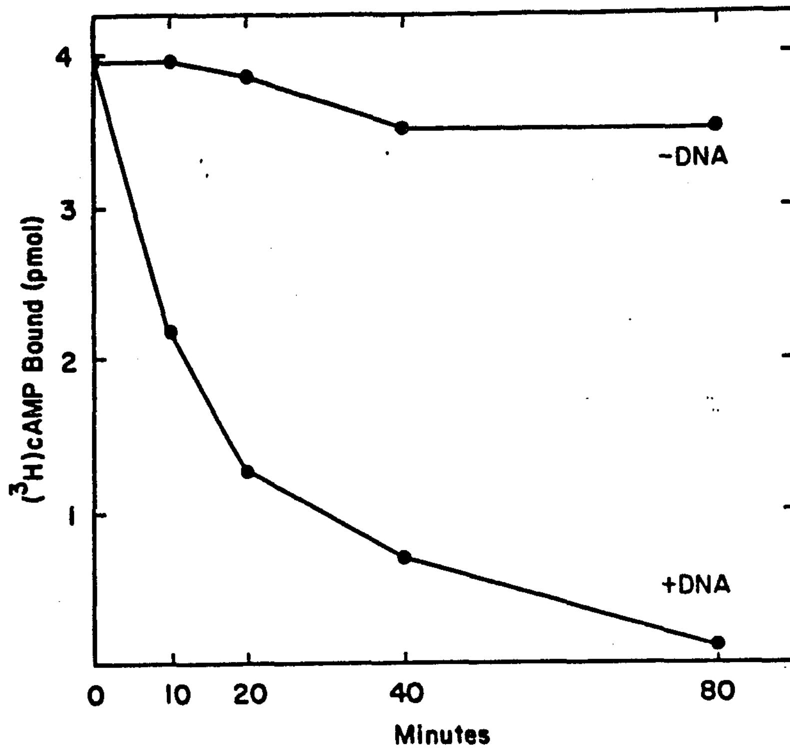

cAMP
CT DNA
Minutes
5
10
20
40

Figure 8: loss of cAMP binding activity after trypsin digestion of CRP in the presence of DNA. The reaction vessel (final volume 100 uL) contained 20 ug of CRP, 16 ug of calf thymus DNA, 10mM Hepes buffer, pH 8.0, 10mM NaCl, 1.0mM EDTA, 0.1mM DTT and trypsin at 1% (w/w). At the indicated time, 15 uL were withdrawn and assayed for ^{3}H-cAMP (3,000 cpm/pmol) binding activity as described by Pastan et al. (1974)



4
3
2
1
(^{3}H)cAMP Bound (pmol)
-DNA
+DNA
0
10
20
40
80
Minutes

Figure 9 : effect of ionic strength on the trypsin sensitivity of the CRP-DNA complex. The reaction mixture contained 20 ug of CRP, 16 ug of calf thymus DNA, 10mM Hepes buffer, pH 8.0, 1.0mM EDTA, 0.1mM DTT, trypsin at 1% (w/w) and NaCl at the indicated concentrations. After 30 minutes at 37°C, 15 uL were removed and assayed for 3H-cAMP (3,000 cpm/pmol) binding activity.

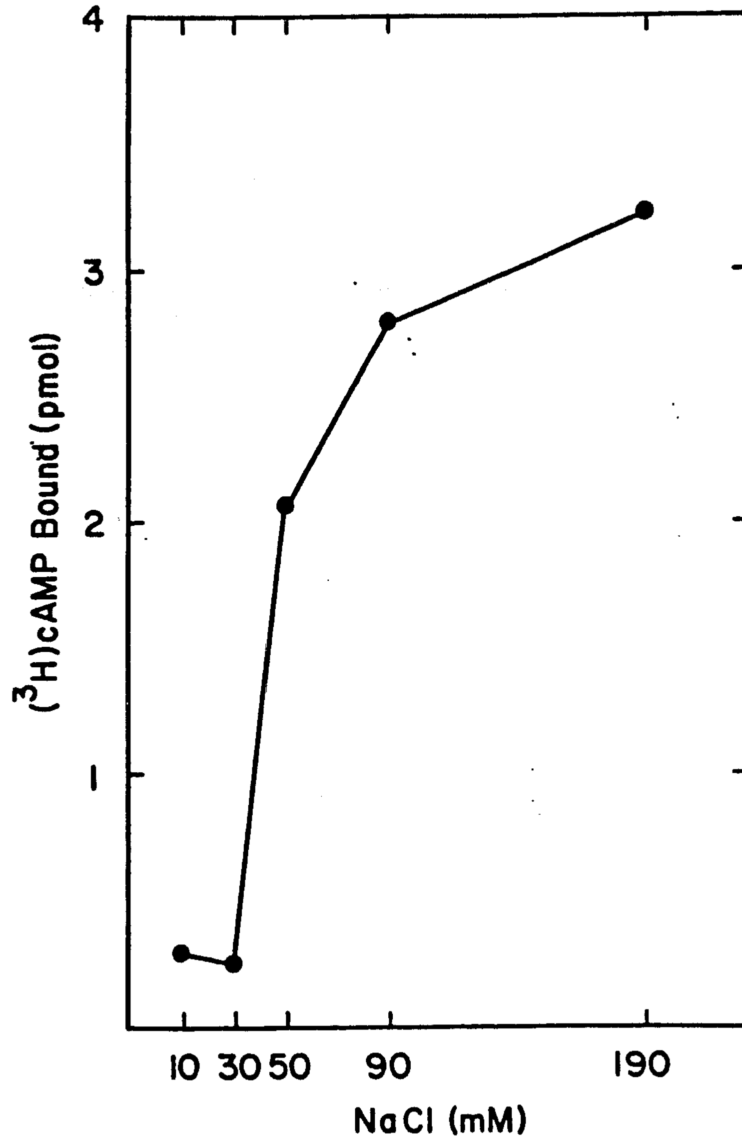
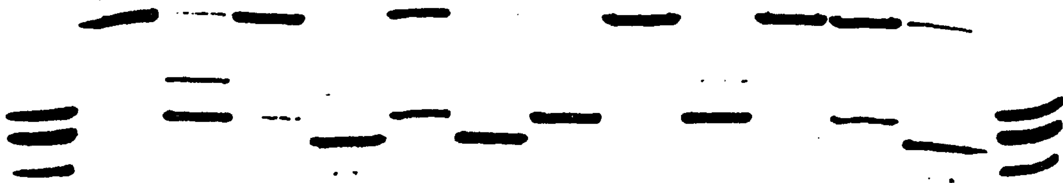

4
3
2
1
(^{3}H)cAMP Bound (pmol)
10 30 50 90 190
NaCl (mM)

Figure 10: digestion of CRP-polymer complex with trypsin. The reaction mixture consisted of 20 ug of CRP, 16 ug of polymer, 10mM Hepes buffer, pH 8.0, 10mM NaCl, 1.0mM EDTA, 0.1mM DTT and 0.1mM cAMP where indicated (final volume 100 uL). Trypsin was used at 1% w/w. After 30 minutes at 37°C the reactions were terminated by the addition of 5 uL of 20mM PMSF. Molecular weight markers in lanes 1 and 14 are: sperm whale myoglobin, 17,800; cytochrome c, 12,600; and bovine pancreas trypsin inhibitor, 6,500.



cAMP	−	+	+	−	+	−	+	−	+	−	+	−
Polymer	−	−	$r(I)_n$		$r(A)_n$		$d(A)_n$		$r(C)_n$		CT DNA	

Figure 11: purification of 10K and 6K fragments of CRP. The fragments were generated and purified as described in "Materials and Methods". Lanes: a) native CRP; b) DNA-CRP digested with trypsin for 30 minutes at 37°C; c) purified 10K fragment; and d) purified 6K fragment. Molecular weight markers in lanes 1 and 8 are: sperm whale myoglobin, 17,800; cytochrome c, 12,600; and bovine pancreas trypsin inhibitor, 6,500.

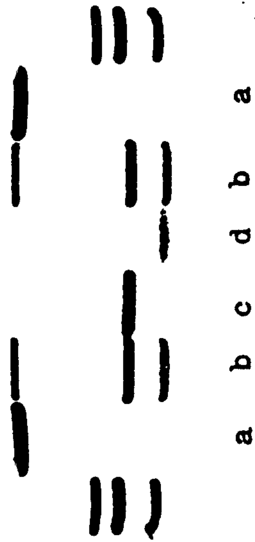

a b c d b a

Figure 12: separation of a mixture of amino acid DABTH derivatives on a Zorbax ODS column (DuPont). Key: D, DABTH-aspartic acid; E, DABTH-glutamic acid; N, DABTH-asparagine; Q, DABTH-glutamine; T, DABTH-threonine; G, DABTH-glycine; Y, DABTH-tyrosine; A, DABTH-alanine; S, DABTH-serine; W, DABTH-tryptophan; M, DABTH-methionine; V, DABTH-valine; P, DABTH-proline; F, DABTH-phenylalanine; L, DABTH-leucine; I, DABTH-isoleucine.

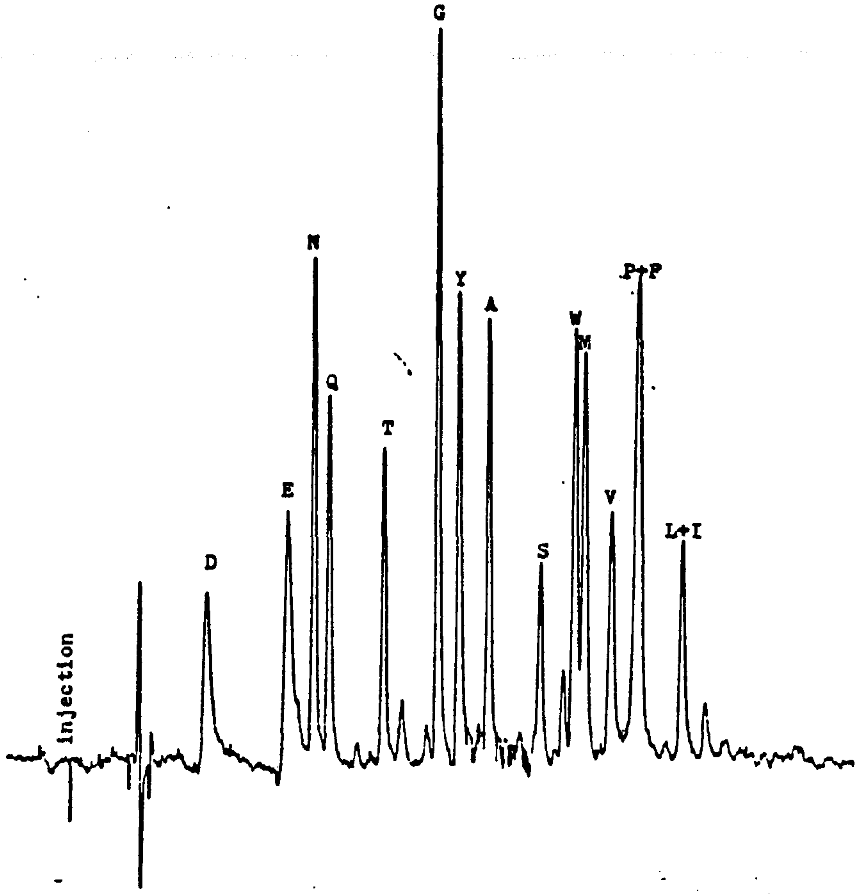

injection
D
E
N
Q
T
G
Y
A
S
W
M
V
P+P
L+I

Figure 13: gradient profile used in the separation of DABTH-amino acid derivatives. The chromatographic conditions were as described in "Materials and Methods". The gradient profile was programmed into the Apple II plus as described in pages 16-30 of the Gilson System Manager manual.

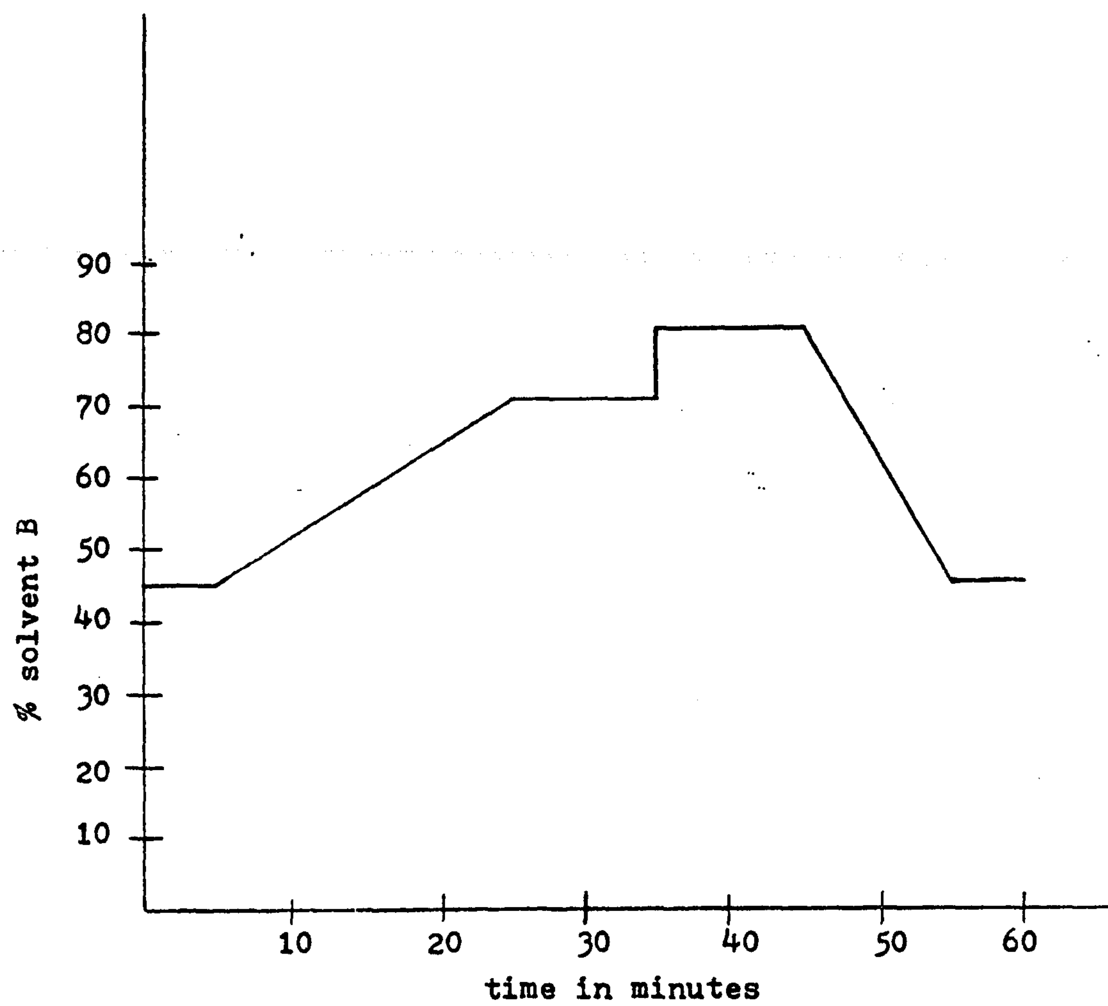
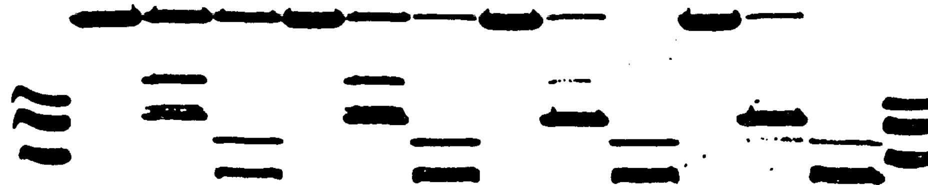
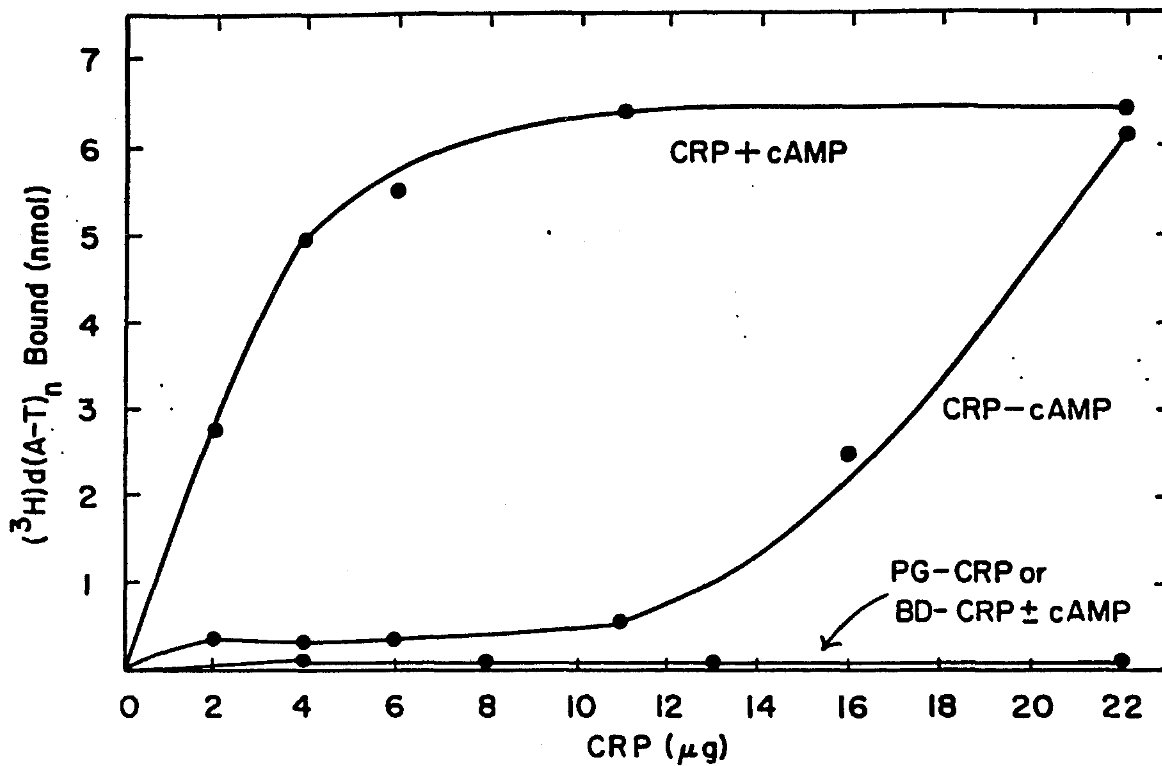

% solvent B
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
time in minutes

Figure 14: time course of digestion of the CRP-heparin complex with trypsin. The reaction tube contained (total volume 100 uL) 20 ug of CRP, 10 ug of heparin, 10mM Hepes buffer, pH 8.0, 10mM NaCl, 1.0mM EDTA, 0.1mM DTT, 0.1mM cAMP where present and trypsin at 1% (w/w). The complex was digested for the times indicated at 37°C. The reaction was terminated with the addition of 5 uL of 20mM PMSF.



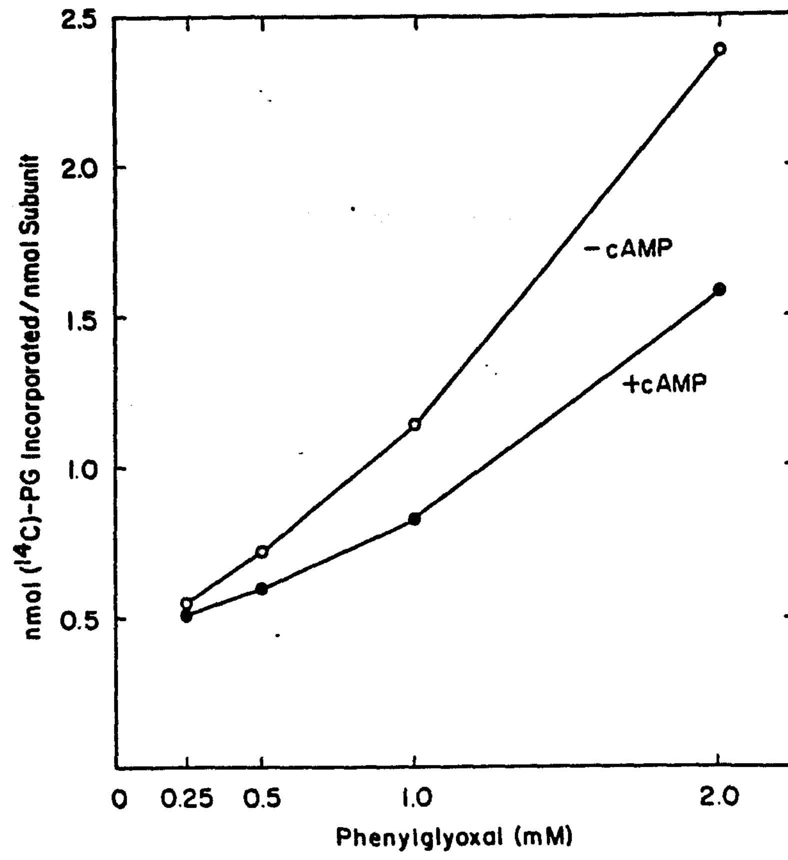
cAMP - + - - + - - + - - + -
HEPARIN - - + - - + - - + - - +
10 20 40 80

Figure 15: $^{3}H-d(A-T)_n$ binding activity of native CRP and CRP modified with phenylglyoxal or butanedione. The reaction mixture consisted (total volume 100 uL) of 40 ug of CRP, 1.0mM phenylglyoxal or 2.5mM butanedione, 50mM sodium borate buffer, pH 8.0 and 0.1mM cAMP where present. After 60 minutes at 37°C, the reaction was terminated with the addition of 6X molar excess arginine. The mixture was dialyzed overnight and then assayed for $^{3}H-d(A-T)_n$ binding activity as described in "Methods".



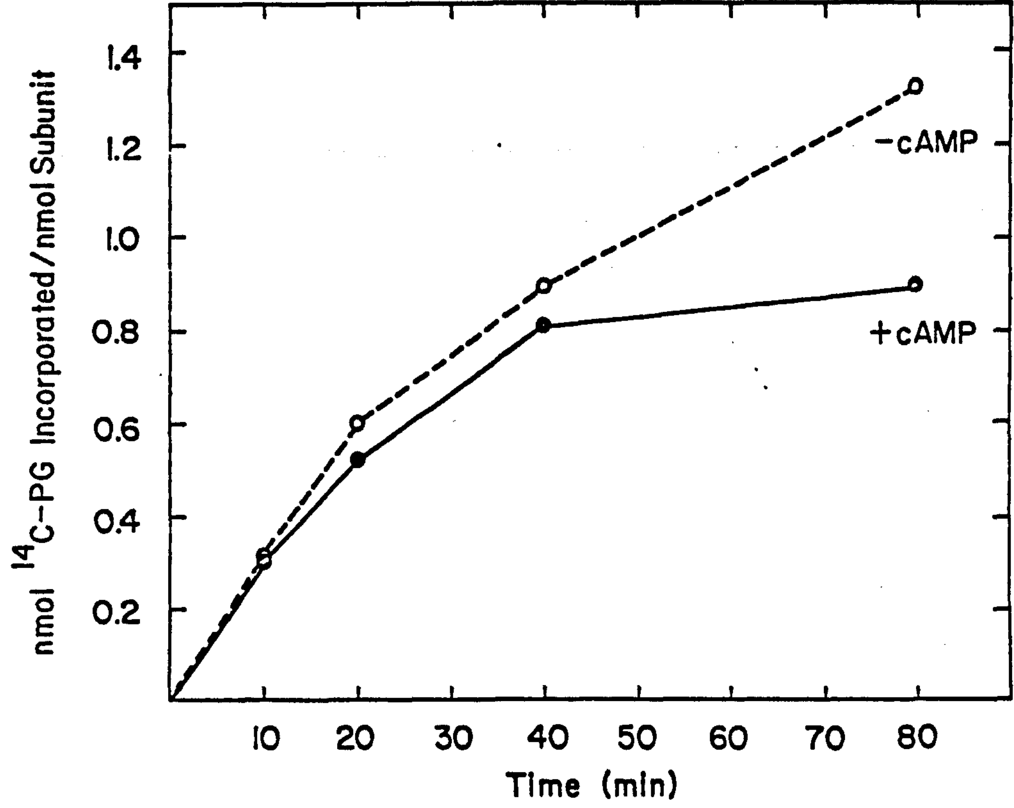
CRP + cAMP
CRP − cAMP
PG−CRP or
BD−CRP ± cAMP
(^{3}H)d(A-T)$_n$ Bound (nmol)
CRP (μg)
0
2
4
6
8
10
12
14
16
18
20
22
1
2
3
4
5
6
7

Figure 16: incorporation of ^{14}C-phenylglyoxal into CRP as a function of ligand concentration. The reaction mixture (final volume of 100 uL) contained 40 ug of CRP, ^{14}C-phenylglyoxal as indicated, 50mM sodium borate buffer, pH 8.0, and 0.1mM cAMP when present. After incubation for 40 minutes at 37°C, the reaction was terminated with the addition of 6X molar excess arginine. The mixture was dialyzed overnight at 4°C and 5 ug of protein precipitated in 0.5 mL of 5% TCA. The precipitate was collected on a nitrocellulose filter and counted in Aquasol II for one minute.



2.5
2.0
1.5
1.0
0.5
nmol (14C)-PG Incorporated/nmol Subunit
−cAMP
+cAMP
0
0.25
0.5
1.0
2.0
Phenylglyoxal (mM)

Figure 17: time course of incorporation of ^{14}C-phenylglyoxal into CRP. Modification reaction conditions were as described in "Methods". For each time point, an aliquot was dialyzed overnight at 4°C. 5 ug of protein were precipitated in 0.5 mL of 5% TCA and the precipitate collected on a nitrocellulose filter. The filter was counted in Aquasol II to determine the counts incorporated into CRP.



nmol 14C-PG Incorporated/nmol Subunit
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
70
80
Time (min)
-cAMP
+cAMP

Figure 18: inactivation of ^{3}H-d(A-T)$_n$ binding activity of CRP with butanedione. The reaction mixture (total volume 100 uL) contained 50 ug of CRP, 50mM sodium borate buffer, pH 8.0, 0.1mM cAMP and the indicated concentration (millimolar) of butanedione. At various time intervals, 10 uL aliquots were removed and assayed for ^{3}H-d(A-T)$_n$ binding activity in the presence of cAMP.

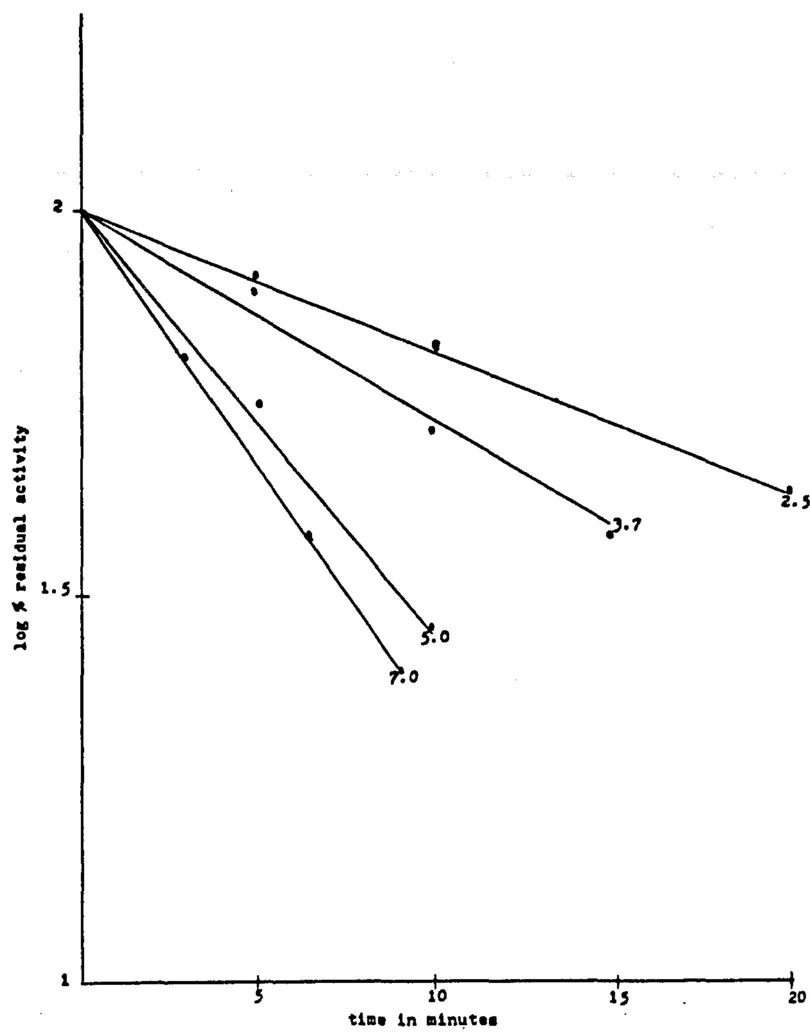

log % residual activity
2
1.5
1
5
10
15
20
time in minutes
2.5
3.7
5.0
7.0

Figure 19: reaction order of CRP inactivation with respect to butanedione concentration. Logarithmic values of $1/t_{\frac{1}{2}}$ were plotted against the log (butanedione) values in millimolar units (log I). Both values were derived from Figure 18.

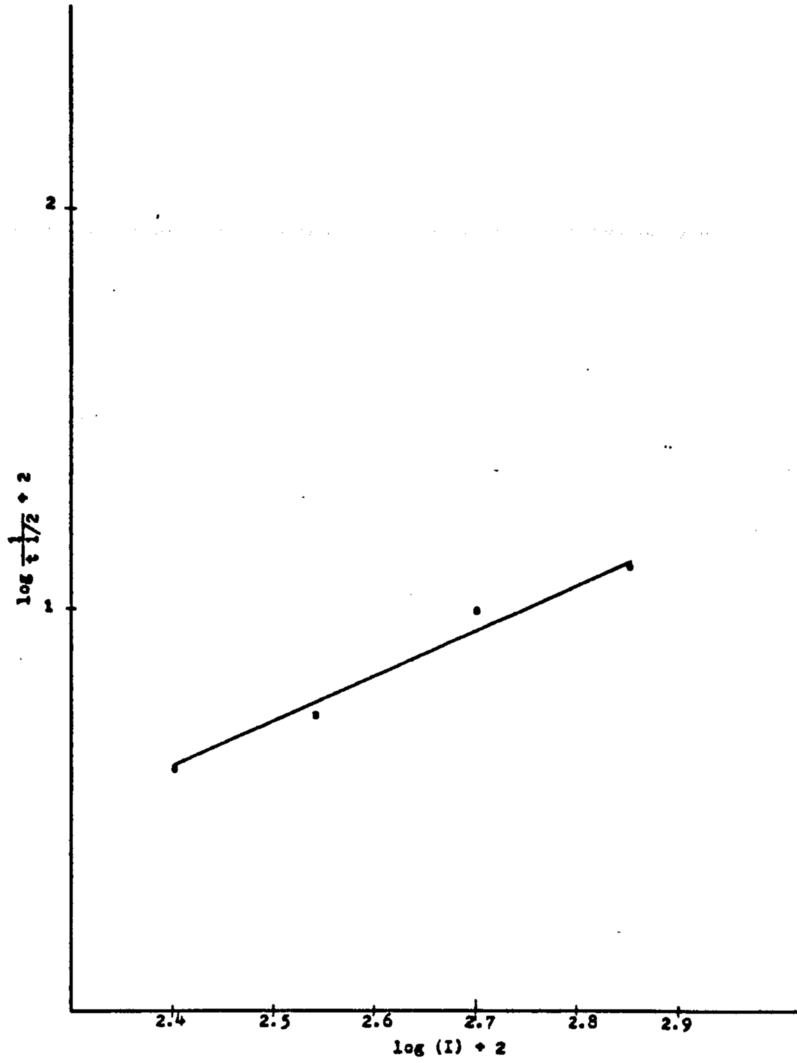
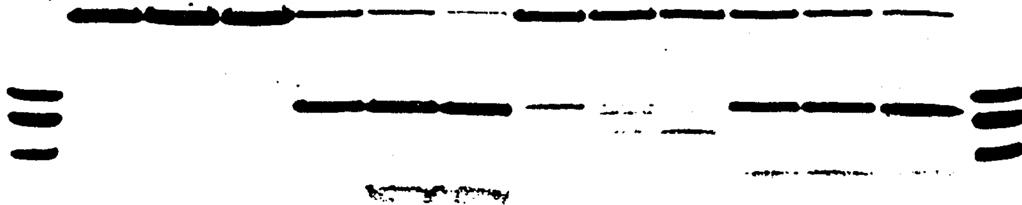
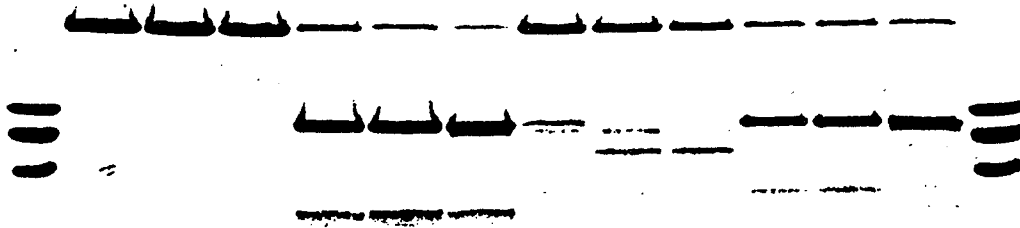

2
1
log $\frac{1}{t\ 1/2}$ + 2
2.4
2.5
2.6
2.7
2.8
2.9
log (I) + 2

Figure 20: chymotrypsin digestion of CRP modified with butanedione. CRP was modified with butanedione in the presence of cAMP. After overnight dialysis, 10 ug of BD-CRP were incubated with the indicated amount of chymotrypsin (w/w) at 37°C for 20 minutes. Control CRP (lanes 2-7) was also incubated at 37°C for 20 minutes in the presence of cAMP and then dialyzed overnight. The hydrolysis reaction mixture consisted of 50mM sodium borate buffer, pH 8.0, 1.0mM EDTA and 0.1mM cAMP where present (total volume of 50 uL). The reactions were terminated with the addition of 5 uL of 20mM PMSF. 30 uL of the reaction mixture were layered on a 15% SDS-polyacrylamide gel.



cAMP	-	-	-	+	+	+	-	-	-	+	+	+
% CHYMO	1	2	4	1	2	4	1	2	4	1	2	4

Figure 21: chymotrypsin digestion of CRP modified with butanedione. Conditions were the same as for Figure 20, except that chymotryptic hydrolysis was for 40 minutes at 37°C.



cAMP	-	-	-	+	+	+	-	-	-	+	+	+
% CHYMO	1	2	4	1	2	4	1	2	4	1	2	4

Figure 22: chemical structure of butanedione-arginine and phenylglyoxal-arginine. The reaction of butanedione with arginine results in the formation of a 4,5-dimethyl-4,5-dihydroxy-2-imidazoline (I) that can be stabilized by the borate ion as shown in II (Riordan, 1973). Di-phenylglyoxal-arginine can exist in two different conformations, III and IV (Takahashi, 1968).

(I)

(II)

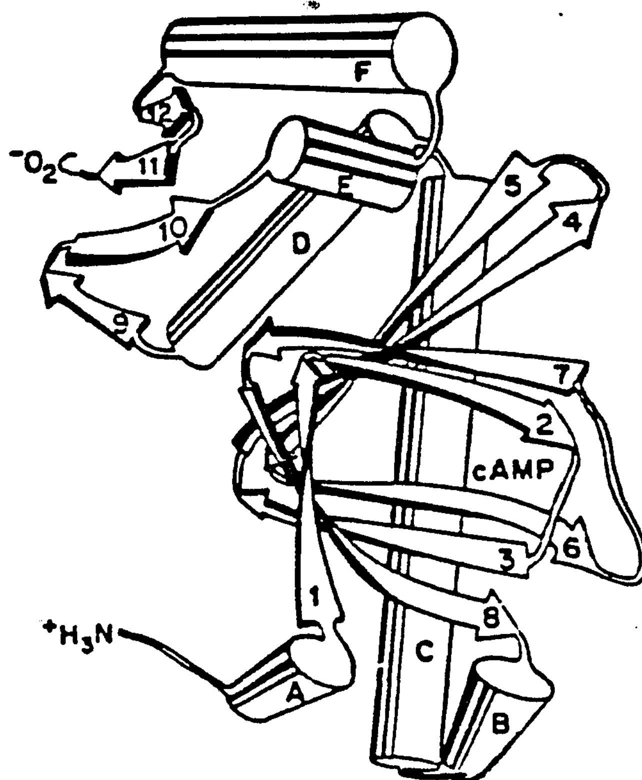
(III)

(IV)

Figure 23: secondary structure of the CRP monomer reproduced from McKay, Weber and Steitz (1982). Alpha-helical regions of the polypeptide are represented as tubes and lettered A through F. Polypeptide segments that are in beta conformation are represented as arrows numbered 1-12.

Helix[a]	Residues	β sheet[b]	Residues	β sheet	Residues
α_A	10–17	β_1	18–25	β_7	81–89
α_B	99–107	β_2	26–35	β_8	90–97
α_C	111–134	β_3	39–43	β_9	156–158
α_D	139–153	β_4	44–52	β_{10}	163–165
α_E	168–176	β_5	58–66	β_{11}	196–198
α_F	180–193	β_6	67–71	β_{12}	202–204

[a] α helix = 80 residues.
[b] β sheet = 75 residues.



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