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The Application of a Reporter Group to α-Chymotrypsin

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THE APPLICATION OF A REPORTER GROUP TO α -CHYMOTRYPSIN

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by

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Approved for publication

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Preface

By instigating discussions between the faculty and students and by offering the tools and knowledge of many fields, Dr. Bronk has made my experience as a graduate student at The Rockefeller Institute one of inestimable value.

The faculty have always been generous with their time and imaginative in answering the queries of a student.

I would especially like to thank Dr. Koshland who in his weekly discussions has encouraged me to express my naive ideas and who has always tried to show me how they may be better formulated and placed in the larger perspective of protein chemistry. He has taught me to apply the tools of organic chemistry to the study of enzymes and to appreciate how complex the structure of these single molecules must be to catalyze biological transformations with such specificity. He has constantly and warmheartedly guided me in all my studies at The Rockefeller Institute.

There are many other faculty members I would like to thank for their encouragements and discussions. Dr. Yphantis taught me much about the physical chemistry of proteins and the art and many applications of the analytical ultracentrifuge. Dr. Mauzerall taught me the theory of chemical kinetics. He also suggested the method used for analyzing the data from the spectrophotometric titrations. Dr. Némethy has encouraged me to make critical physical interpretations and Dr. Yankeelov helped me with the synthesis of organic compounds. Dr. Némethy and Dr. Yankeelov made my work in the laboratory pleasurable.

I would like to thank Dr. Jaffe for his assistance with the infrared analyses and for generously making the equipment in his laboratory available to me.

I would like to thank the members of Dr. Koshland's laboratory at the Brookhaven National Laboratory for pleasant, educational summers. I profited especially from discussions with Dr. D. H. Strumeyer and Dr. H. Weiner. Mr. H. G. Latham was always willing to help me with unfamiliar equipment and performed some of the amino acid analyses for me.

I would like to thank Miss J. Vogel for skillfully drawing many of the figures and tables.

Abstract

A reporter group method was developed in order to obtain information about small changes in the environment at specific positions in protein molecules. In this method one environmentally sensitive group is introduced into a specific position in the protein so that small changes induced by substrates or modifiers can be followed.

In this study the synthesis of a potential reporter group reagent, 2-bromoacetamido-4-nitrophenol, was accomplished. This reagent has a moiety which is sensitive to the polarity of the environment and to pH, and which reports changes in its environment to an appropriate detector. It contains a second moiety which reacts with methionine residues. This potential reporter group was attached to chymotrypsin by a covalent bond to a methionine residue. It was demonstrated that substrates perturb the spectrum of the reporter-modified chymotrypsin and that the spectral perturbations have characteristics related to a tentative but consistent interpretation of changes in the absorption spectrum of the reporter group in terms of the environment of the group.

The applications of reporter groups to the study of conformational changes of proteins and to the study of the three-dimensional orientations of substrates at the active sites of the proteins are discussed.

Abbreviations

ATEE	Acetyl-L-tyrosine ethyl ester
CT	Bovine pancreatic α-chymotrypsin
CT-(R_1-Met_3)	α-Chymotrypsin with the reporter group, R_1, attached to the methionine three residues from the active serine residue.
DFP	diisopropyl fluorophosphate
K_m	apparent Michaelis constant except when otherwise indicated
PMSF	phenylmethyl-C^{14}-sulfonyl fluoride
R_1Br	2-Bromoacetamido-4-nitrophenol, Structure I page 10
R_1H	2-Acetamido-4-nitrophenol
TCA	trichloroacetic acid
V_m	maximal velocity

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Section I

Introduction

INTRODUCTION

Great progress has been made in the last two decades in the correlation of structure and function of proteins. X-ray crystallography coupled with sequence determination has made it possible to delineate the three-dimensional structures of proteins such as hemoglobin (Perutz et al., 1960; Perutz, 1960) and myoglobin (Kendrew et al., 1960; Kendrew, 1962). Modification of amino acid residues by reagents that are specific and stoichiometric has led to the identification of many residues as essential or nonessential for enzymatic activity (Balls and Jansen, 1952; Gundlach, Stein, and Moore, 1959a; Crestfield, Stein, and Moore, 1964a,b; Hirs, 1962; Koshland, Strumeyer, and Ray, 1962a; Schoellmann and Shaw, 1963). The application of these techniques will no doubt yield both the three-dimensional structures of many proteins and the positions in these structures of the essential amino acid residues.

Although these techniques may lead to much valuable information on the roles of the amino acid residues in the binding and catalysis of substrates by enzymes, further information will be needed to complete the correlation of the structures of proteins with their functions. For example, information describing the orientations of the various nonreactive residues at the active site with the various parts of the substrate molecule and describing the role of the conformational changes in enzymes during the binding and catalysis of substrates, will be needed.

To aid in the solution of these problems a new technique was developed which involves the introduction of an environmentally sensitive group into specific positions of a protein molecule. This group is designed to report changes in its environment by transmitting a signal to an appropriate laboratory detector. This thesis describes the application of this technique to bovine pancreatic α-chymotrypsin.

A. Background and Rationale

The pertinent literature and some of the reasoning that lead to this work will be summarized in this section.

α-Chymotrypsin The zymogen of the digestive enzyme, chymotrypsinogen, is secreted by the pancreas into the duodenum of the small

intestine where it is activated by trypsin and chymotrypsin. The active enzyme cleaves peptides and proteins at the carboxyl side of aromatic amino acid residues such as phenylalanine, tyrosine, and tryptophan. It also hydrolyzes analogous esters and amides.

α-Chymotrypsin has three peptide chains which are covalently linked by disulfide bonds. The A-chain has 13 amino acid residues, the B-chain 131 residues, and the C-chain 98 residues. The primary structure of chymotrypsin has recently been determined by Hartley (1964a, 1964b[1]) and Keil, Prusik, and Šorm (1963). A partial sequence of α-chymotrypsin which includes the amino acid residues that have been identified as part of the active site and the two methionine residues of chymotrypsin are shown in Figure 1.

Of the 28 serine and 2 histidine residues of α-chymotrypsin, one serine on the C-chain and one histidine residue on the B-chain have been identified as essential for enzymatic activity. Diisopropylfluorophosphate inactivates chymotrypsin by reacting with a single serine residue (Balls and Jansen, 1952; Schaffer, May, and Summerson, 1953). The loss of a single histidine residue by photooxidation results in the complete loss of activity (Koshland et al., 1962a). Substrates or substrate-like reagents that bind to the active site have been covalently bonded to these essential residues indicating that these residues are structurally at the active site of chymotrypsin. A serine residue was acylated by the substrate, para-nitrophenyl acetate (Oosterbaan and Van Andrichem, 1958), and the histidine nearest the carboxy-terminal residues reacted with the chloromethyl and the bromomethyl ketone of N-tosyl-L-phenylalanine (Schoellmann and Shaw, 1962, 1963; Ong, Shaw, and Schoellmann, 1965).

Modification of some other amino acid residues of chymotrypsin resulted in enzymes that were fully active or had only a slight decrease in their rates of hydrolysis. The functional groups of these amino acids are not essential for catalysis to occur. These amino acid residues will,

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1 The sequence determined by these workers will be used throughout this paper.

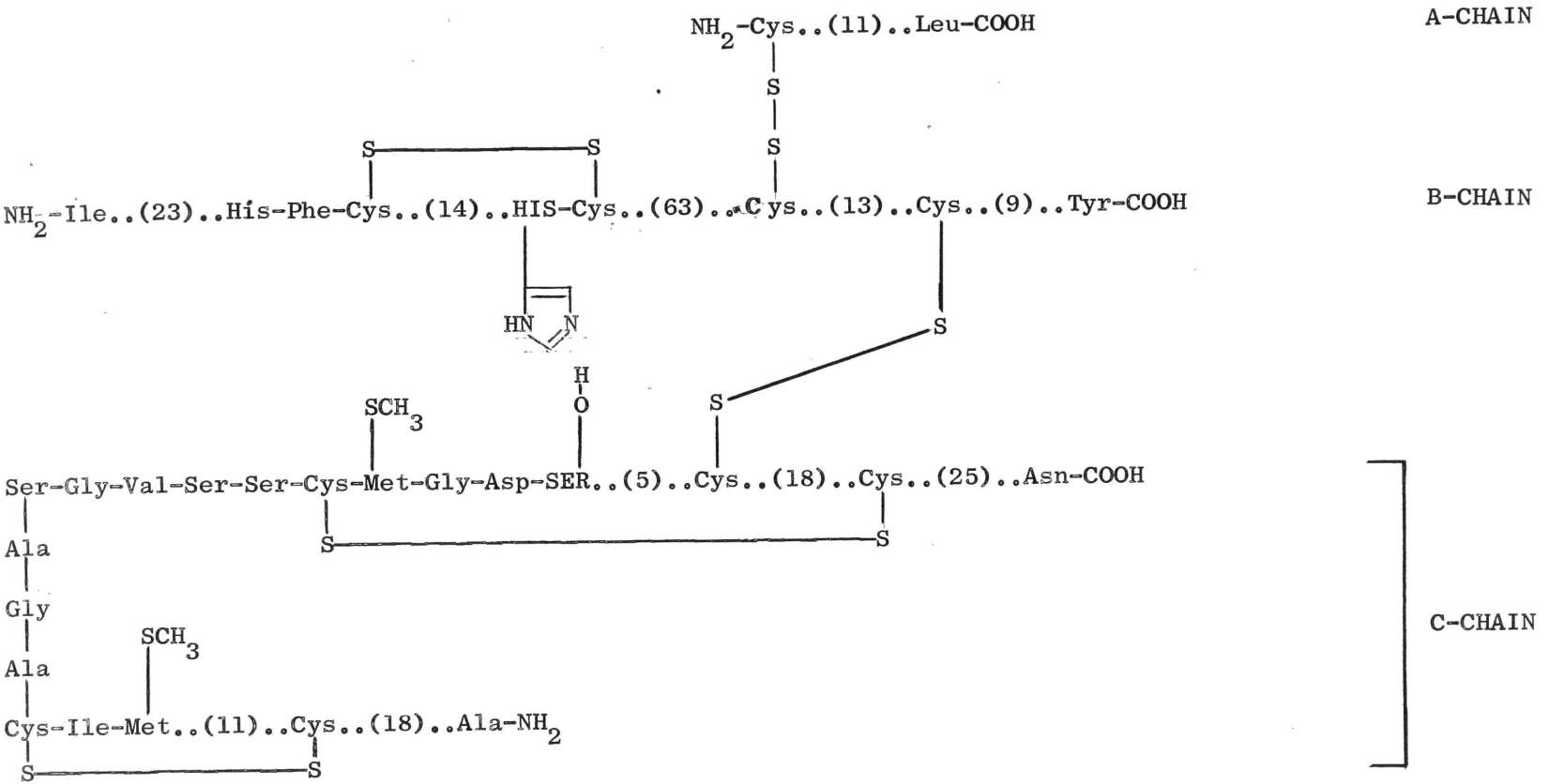

Figure 1 A partial sequence of α-chymotrypsin

The sequence is that reported by Hartley (1964b). The active serine in the C-chain and the active histidine in the B-chain are given with their functional groups. The functional groups of the methionines 3 and 15 residues from the active serine, and of the disulfide bridges are also given. The number of residues not shown are indicated in parenthesis.

therefore, be referred to as nonessential residues. These residues include all 14 of the lysine residues of α-chymotrypsin, 7 or 8 of the 8 tryptophan residues and the 2 methionine residues. The lysine residues were modified with O-methylisourea (Chervenka and Wilcox, 1956b) and with fluorodinitrobenzene (Massey and Hartley, 1956) without changing the activity of the enzyme. The tryptophan and methionine residues were oxidized with N-bromosuccinimide (Viswanatha and Lawson, 1961) and peroxide (Koshland et al., 1962a), respectively, with only a slight decrease in the activity of the enzyme. In addition, modification of the amino-terminal half cystine residue of the A-chain with carbon disulfide (Chervenka and Wilcox, 1956a) and of the amino-terminal alanine residue of the C-chain with fluorodinitrobenzene (Massey and Hartley, 1956) and of the carboxy-terminal tyrosine residue of the B-chain with iodine (Glazer and Sanger, 1964) leads to no loss of activity. Iodination of the 3 other tyrosine residues was also shown to lead to little or no loss of activity (Filmer and Koshland, 1964). Thus far insufficient modification studies of the carboxyl groups in chymotrypsin have been made to determine if these groups are essential for activity. One study indicates that some of these residues are not essential since modification of an average of 3.5 of the 18 carboxyl groups of chymotrypsin by diazoacetamide results in only a small decrease in the rate of catalysis (Doscher and Wilcox, 1961). Modification studies have, as yet, failed to identify any amino acid residues, other than the single serine residue at position 195 and the histidine residue at position 57 in Hartley's sequence of chymotrypsinogen, as essential for enzymatic activity.

A hypothesis for the catalytic roles of the essential serine and histidine residues that is consistent with the accumulated data is that histidine acts as a general base catalyst abstracting a proton from serine during acylation (Bender, 1962; Anderson, Cordes, and Jencks, 1961; Cunningham, 1957). The serine hydroxyl in a concerted reaction can then act as a nucleophile and attack the carbonyl carbon of the ester or amide bond in the substrate. During deacylation histidine may remove a proton from the water molecule that displaces serine.

Modification of methionine residues Previous modification studies of methionine residues of α-chymotrypsin are similar to the one described

in this thesis.

The reaction of iodoacetic acid with methionine to produce the carboxymethyl-sulfonium salt of methionine has been studied by Gundlach, Moore, and Stein (1959b). They found that the extent of the reaction was pH independent from pH 4 to 8.5. Since the methionine sulfonium salt decomposes under the conditions normally used for acid hydrolysis of proteins, the extent of modification of methionine residues in proteins cannot be determined directly. Performic acid oxidation of modified proteins produces the acid stable methionine sulfone residue from unmodified methionine residues. Therefore, the amount of unreacted methionine residues can be determined by performic acid oxidation followed by acid hydrolysis and analysis for methionine sulfone (Neumann, 1960; Moore, 1963).

Likewise, the extent of methionine sulfoxide formation by oxidation of methionine residues could not be determined by acid hydrolysis since the sulfoxide residues revert to methionine in acid solution (Ray and Koshland, 1960). Methionine sulfoxide does not, however, revert under the conditions of barium hydroxide hydrolysis, and can be analyzed after basic hydrolysis. They can also be analyzed as the methionine sulfone by the method of Neumann, Moore, and Stein if the unmodified methionine residues are protected by alkylation with iodoacetic acid before oxidation with performic acid.

Iodoacetic acid reacts with the uncharged form of imidazole and amino groups, so that selective reaction with methionine or cysteine residues usually occurs at pH values below 6. However, it was found for ribonuclease that the conformation of the protein causes both an unusual reactivity of the histidine residues and protection of the usually reactive methionine residues between pH 5.5 and 6. At pH 4 this conformation no longer exists and methionine residues are alkylated while histidine residues are not (Gundlach, Stein, and Moore, 1959a).

Koshland, Strumeyer, and Ray (1962a) took advantage of the low reactivity of amino and imidazole groups below pH 4 in studying the reaction of iodoacetic acid with chymotrypsin. Since chymotrypsin contains no sulfhydryl groups, only reactions with methionine residues occurred at pH 3. They found that at pH 3 both iodoacetic acid and peroxide modify only one of

the two methionine residues of α-chymotrypsin. Both methionine residues are accessible in 8 M urea. The methionine residue that is accessible when α -chymotrypsin is in its normal configuration was referred to as the "outside" methionine and the other as the "inside" methionine. In the primary sequence one methionine is three residues away from the active serine and the other fifteen. They will be referred to as Met_3 and Met_{15} in this thesis.

Modification of the outside methionine residue with iodoacetic acid or oxidation with peroxide leads to only a partial loss of activity as mentioned previously. The partial loss in activity with the oxidized methionine residue was found to increase the apparent Michaelis constant (K_m) which can be related to a decrease in the binding affinity of the substrate for the enzyme. The maximal velocity of the reaction (V_m) was not changed, indicating that the configuration responsible for hydrolysis was not affected by this modification.

The position of the outside methionine residue was determined by Koshland, Strumeyer, and Ray using the end group method of Stark and Smyth (1963) and the selective cleavage procedure at methionine residues of Gross and Witkop (1961; Lawson et al., 1962). In the latter procedure, treatment of peptides and proteins with cyanogen bromide results in cleavage at the carboxyl side of methionine residues with the formation of a homoserine lactone end-group. No cleavage occurs if the methionine residue has been previously modified to the sulfoxide residue. α-Chymotrypsin cleaved by cyanogen bromide was found to have 5 amino-terminal residues: a glycine and an isoleucine residue that were on the carboxyl side of Met_3 and Met_{15}, respectively, and a half cystine, an isoleucine, and an alanine residue that are the amino-terminal residues of the A, B, and C-chains (Figure 1). If the outside methionine residue were Met_3, the oxidized cyanogen bromide-treated enzyme would be expected to yield one less glycine residue and if it were Met_{15}, one less isoleucine residue. In the actual experiment the oxidized enzyme was found to have 1 half cystine, 2 isoleucine and 1 alanine residues. Thus, the methionine three residues from the active serine is stoichiometrically modified, and is the only residue modified. This peroxide-oxidized enzyme lost no further activity when treated with iodoacetic acid at pH 3.

Therefore, it was concluded that the same methionine residue, Met_3, was modified by peroxide and iodoacetic acid treatment.

Binding of substrates by α-chymotrypsin Descriptions of the orientations of substrates at the active site and their relative contributions to binding are essential for a complete understanding of the relationships of enzyme structures to their functions. Niemann and co-workers have postulated that a substrate can bind in several different ways to the active site but that only certain of these binding modes (productive modes) can lead to reaction products (Hein and Niemann, 1962a, b; Niemann, 1964). The "nonproductive" modes of binding are competitive with the productive modes of binding. These workers also postulate that there are three areas at the active site that contribute to binding and a fourth area occupied by the H on the asymmetric carbon of a normal substrate. The binding loci interact with the α-acylamino group (locus 1), the amino acid side chain (locus 2), and the carboxylate function (locus 3) of a normal substrate. Qualitative judgements about the orientation of substrates at the postulated binding loci and the contributions of these binding modes to catalysis were possible when the kinetic constants of the substrates were interpreted as representing several rather than one mode of binding. Interactions which appeared to determine the dominant modes of binding were interactions between bulky groups and locus 2. A requirement for a productive mode of binding was an interaction between the carboxylate function and locus 3. Binding of the α-acylamino groups at locus 1 appeared to increase the catalytic rate constant or in other words to orient the substrates so that they were in proper alignment with the catalytic groups on the enzyme. Bulky groups at the H-locus had the opposite effect and decreased the catalytic rate constant. It has been shown for another enzyme, β-amylase, that substrates do bind in several ways to the active site, and that only one of these binding modes is productive (Thoma and Koshland 1960).

Role of conformational changes It is becoming increasingly apparent that conformational changes play a vital role in both the specificity function and catalytic function of enzymes. The "induced fit" theory was proposed by Koshland (1958) to explain anomalies in the specificity exhibited by certain enzymes. For instance, compounds that bind to the surface of enzymes but are smaller than the analogous substrates react extremely slowly

if at all. Koshland proposed that only a substrate can cause changes in the conformation of the enzyme such that the catalytic groups will be brought into proper alignment for catalysis to occur. Evidence for this theory has come from many sources such as, (1) the competitive inhibition of β-amylase action by cyclohexa- and cycloheptaamylose and the internal segments of starch molecules (Thoma and Koshland, 1960), and (2) substrate-induced changes in the SH reactivity and fluorescence properties of phosphoglucomutase (Koshland, Yankeelov, and Thoma, 1962b).

A different line of evidence for the role of protein conformational changes arises from the effects of end-product metabolites on the initial enzyme in a biosynthetic pathway. The end-product appears to inhibit the latter by binding to a regulatory or allosteric site that is distinct from the active site of the enzyme (Gerhart and Pardee, 1961, 1962; Changeux, 1961). Monod and co-workers proposed (Monod, Changeux, and Jacob, 1963; Changeux, 1963, 1964) that the regulatory metabolite induces a conformational change in the protein which alters the active site of the protein. They also suggest that the alteration is due to a change in the interactions between the subunits of the protein. Gerhart and Pardee (1962, 1963; Gerhart 1964) have similarily proposed that the binding of cytidine triphosphate to the regulatory site of aspartate transcarbamylase decreases the affinity of the active site for the substrate by strengthening the subunit interactions of the enzyme and thus distorting its active site.

Spectral method for detecting conformational changes One of the many methods for detecting conformational changes in enzymes is to observe spectral perturbations of absorbing groups. This method was modified for our studies. Previously, changes in the ultraviolet absorptions of protein residues such as tyrosine, tryptophan, and phenylalanine have been followed in order to detect conformational changes during the activation of zymogens or the denaturation of proteins by urea, pH changes, or proteolytic enzymes (reviewed by Wetlaufer, 1962). Similarily, changes in the fluorescence of the tyrosine and tryptophan residues have been used to follow conformational changes of proteins in urea solutions (Weber, 1960). The tryptophan spectrum of α-chymotrypsin was found by Wootton and Hess (1962) to be perturbed by the formation of certain acyl-chymotrypsin complexes. A change in the

fluorescence spectrum of phosphoglucomutase was used to detect substrate-induced conformational changes of this enzyme (Koshland, 1963; Koshland et al., 1962b).

Changes in the absorption of a dye that absorbs in the visible region outside the absorption bands of protein have been used to detect general conformational changes during the acid denaturation of bovine plasma albumin (Williams and Foster, 1960). Recently, the fluorescence of a non-specifically bound dye was used to study the conformation of Bence-Jones proteins (Gally and Edelman, 1965).

Rationale for the development of the "reporter group" method

The "reporter group" method was developed in order to obtain information about small changes in the environment at specific positions in protein molecules. Previous modification studies of proteins had demonstrated that it was possible to modify individual amino acid residues and to follow conformational changes of proteins with dyes which absorb in the visible region outside the protein absorption bands. Our method differs from previously developed spectral methods for following conformational changes in that one environmentally sensitive group or "reporter group" is introduced into a specific position in a protein molecule making it possible to measure small changes induced by substrates or modifiers, rather than gross unfolding or denaturation phenomena.

Two situations in which a reporter group might register environmental changes are shown in Figure 2. In one the reporter group is placed next to the active site so that direct interaction with the substrate[2] is possible. In the other the reporter group is placed at a point distant from the active site. In this case direct contact of the reporter group with the substrate is excluded but substrate-induced conformational changes may trigger changes in the protein conformation in the neighborhood of the reporter group.

A study of direct interactions of substrates with reporter groups may be helpful, for example, in describing the three-dimensional orientation of the parts of the substrate with respect to the various amino acid residues

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2 The word "substrate" will be used generally to indicate both substrates and inhibitors that are absorbed at the active site.

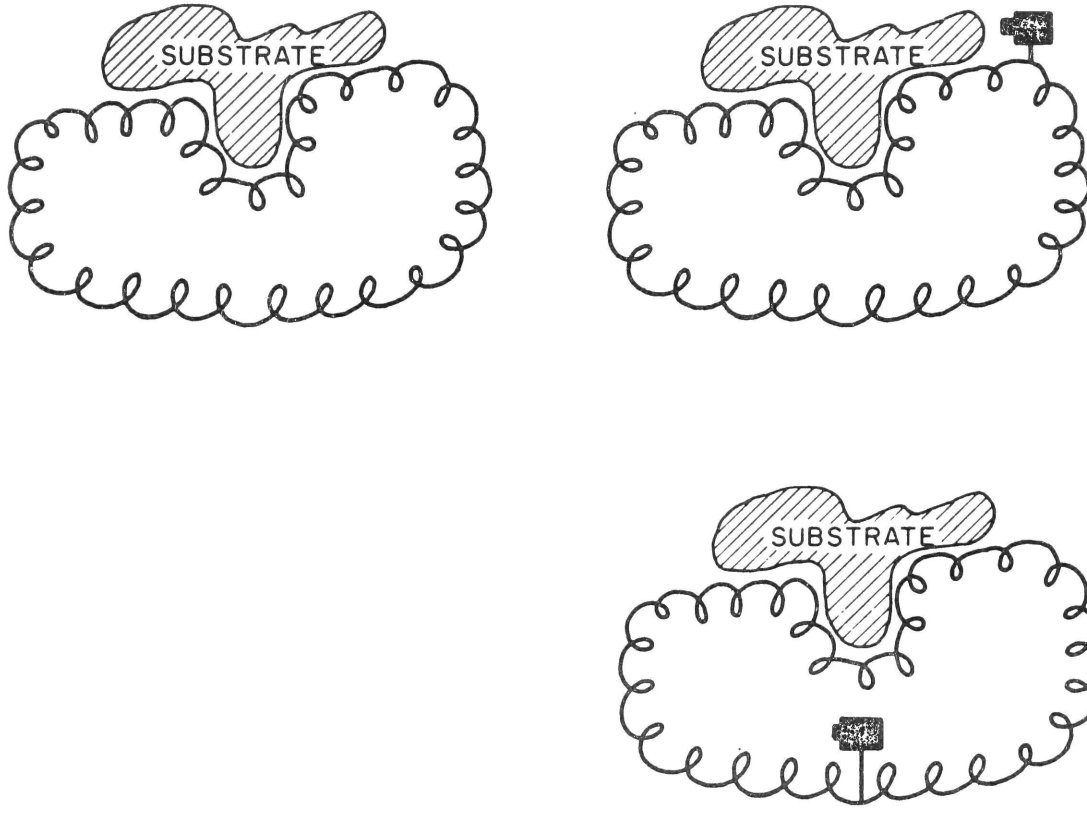


Figure 2 Schematic enzyme with substrate and reporter groups

Schematic representation of an enzyme-substrate complex in native protein and in proteins containing reporter groups (solid black area). The upper right figure shows a reporter group adjacent to the substrate binding area and, the lower right figure, a reporter group distant from the substrate binding area.

at the active site. This problem of determining the orientation of a substrate is illustrated in Figure 3 for several alignments of a substrate on a hypothetical surface (hatched area with the position of 5 amino acids designated by A, B, C, D, and E) of an enzyme. If E represents an essential catalytic amino acid residue, such as the serine residue of chymotrypsin, then the susceptible bond of the substrate (here represented as being near T) must be in contact with E for hydrolysis to occur.

In order to know the three-dimensional alignment of a substrate at the active site of an enzyme, the positions of R and S must also be determined. Even if R is shown to be near a specific residue such as A, there are still two possibilities for the location of S as illustrated by parts a and b of this figure.

If a reporter group were placed near D then a substrate that binds as in Figure 3a would be in contact with a bulky S moiety of the substrate. A small S moiety might not interact with a reporter group at D. By studying several substrates it might be established that the S moiety is in position D. Care must be used in drawing conclusions since modifications of the substrate or of the amino acid residues near the active site may lead to different orientations of the substrate. However, a description of the orientations of substrates on a modified enzyme may still be helpful in understanding the properties that convey enzyme specificity. Placement of reporter groups at several positions at the active site may also lead to a precise description of the orientations of substrates on a native enzyme. Descriptions of substrate orientations are an essential feature in correlating the structure of an enzyme with its function.

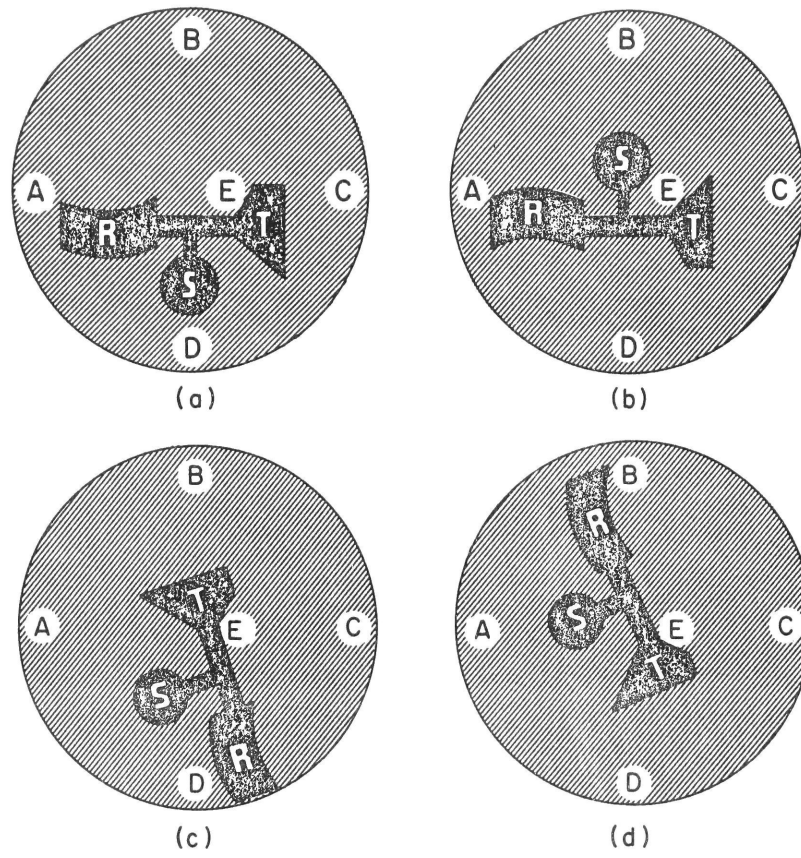


Figure 3 Orientations of a substrate on a hypothetical enzyme surface

A, B, C, D, and E represent positions of amino acid residues. All orientations place an essential residue E adjacent to the T moiety of the substrate. The positions of the substrate in (b) and (c) are achieved by a 180^{o} out-of-plane rotation of that in (a), whereas in (d) the position is achieved by movement of the substrate only within the plane of the enzyme surface.

Section II

Materials and Methods

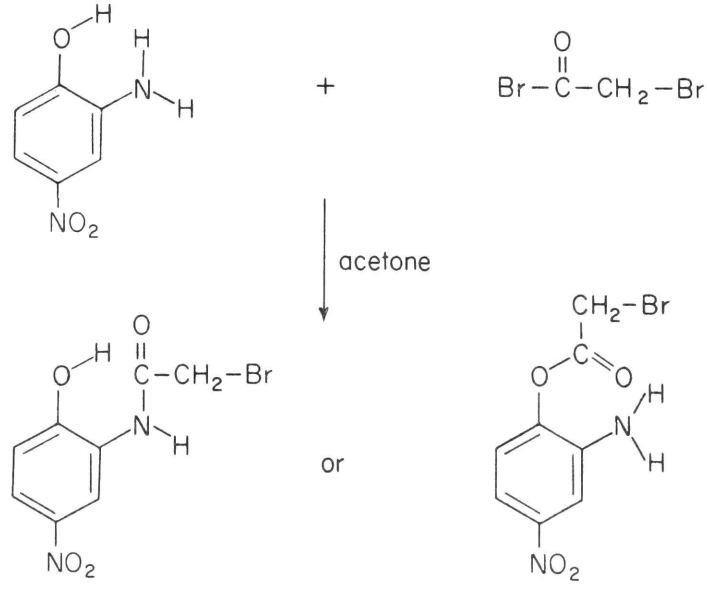
METHODS AND MATERIALS

A. Chemical Synthesis

2-Bromoacetamido-4-nitrophenol 2-Bromoacetamido-4-nitrophenol (R_1Br) was prepared by the procedure used by Newbery and Phillips (1928, p 3049) in the preparation of 2-chloroacetamido-4-nitrophenol. Dropwise addition of 9.25 ml (0.106 mole) of bromoacetyl bromide to 10 g (0.065 mole) of 2-amino-4-nitrophenol (recrystallized from H_2O, mp 141-142.5°) in 80 ml acetone resulted in the concomitant precipitation of crystals. During the addition of bromoacetyl bromide the flask was shaken by hand and cooled when necessary in an ice bath. The crystals were filtered from the cooled solution and recrystallized three times from ethanol (analytical grade 95%), mp 207-208.5° (sealed tube), sublimation range, 193-208° (copper block); and again from ethanol, mp 212-213° (sealed tube).

Analysis: $C_8N_2O_4BrH_7$; calculated: C, 34.93; N, 10.19; Br, 29.05; H, 2.565; found: C, 35.31; N, 10.01; Br, 29.91; H, 2.62.

The elemental analysis is consistent with both Structures I and II (see below). The absolute structure was established from the infrared absorption pattern (Figure 4, Table I).



2-Acetamido-4-nitrophenol 2-Acetamido-4-nitrophenol (R_1H) was prepared according to the method of Hewitt and King (1926, p 823). Two grams (0.013 mole) of 2-amino-4-nitrophenol (mp 141-142.5°) were dissolved in about 16 ml of absolute ethanol. 1.98 ml (0.021 mole) of acetic anhydride were added at room temperature. The precipitate was recrystallized twice from absolute ethanol, mp 272-273° (copper block), 277° (sealed tube) (literature mp 279-280° , Hewitt and King, 1926).

The infrared absorption pattern was similar to that for R_1Br and is given in Table 1.

Infrared Analyses To determine whether 2-bromoacetamido-4-nitrophenol (I) or 2-amino-4-nitrobromoacetylphenol (II) was prepared by the reaction of bromoacetyl bromide with 2-amino-4-nitrophenol, the infrared spectrum of the product was examined in the region of NH and OH vibrations. The absorption spectra of 2-acetamido-4-nitrophenol and 2-amino-4-nitrophenol were also examined.

R_1Br has a sharp absorption band at 3360 cm^{-1} (Figure 4) and a broad absorption band from 3100 to 3000 cm^{-1}. R_1H has a similar absorption pattern, — a sharp absorption band at 3408 cm^{-1} and a broad absorption band from 3000 to 2850 cm^{-1} (Table I). Since it is expected that both Structure I and Structure II would have at least two absorption bands in the region for OH and NH stretching vibrations (3650-3300 cm^{-1}) these data can only be explained by a hydrogen bond between the hydroxyl hydrogen and the carbonyl oxygen of Structure I or the amine hydrogen and carbonyl oxygen of Structure II. By comparing the infrared data with a few examples from the literature we can choose between these structures.

Examples of shifts in the NH and OH stretching frequencies due to inter- and intramolecular hydrogen bonding are given in Table II. In the mixture of phenol and N,N-dimethylacetamide in a nonpolar solvent (CCl_4) the unbonded phenolic OH absorption (3610 cm^{-1}) is shifted by 320 cm^{-1} to a shorter frequency by intermolecular hydrogen bonding with the carbonyl group (Mizushima et al., 1955). Even shorter frequencies are observed when favorable intramolecular hydrogen bonding occurs as in methylsalicylate (Tsuboi, 1952). Ortho or para electron withdrawing groups like chlorine further increase the strength of the hydrogen bond so that a shift of 650 cm^{-1} to a shorter frequency is observed for 3,5-dichloromethylsalicylate (Hoyer, 1956). The nitro group

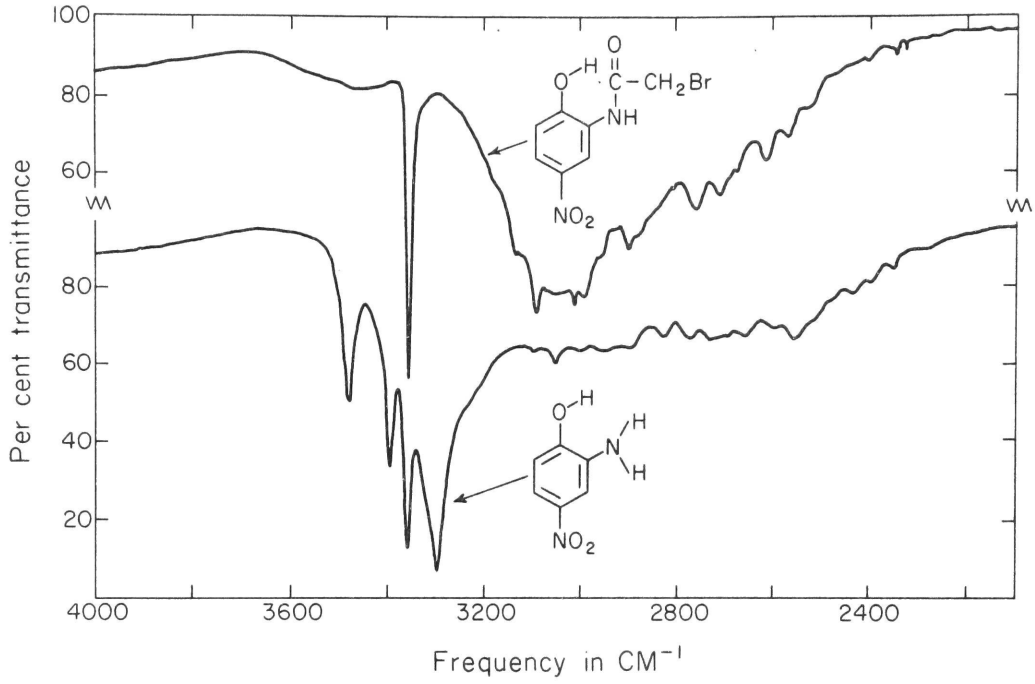
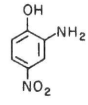
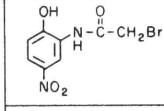
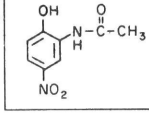


Figure 4 Infrared absorption of R_1Br and the starting material, 2-amino-4-nitrophenol, in the region of the NH and OH stretching frequencies

Measurements were made of the solid phase in KBr pellets with a Perkin-Elmer 221 with a NaCl prism grating interchange.

Table I

Experimental infrared absorption frequencies

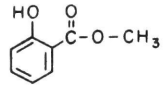
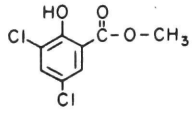
Compounds	OH stretching frequencies in CM^{-1}			NH stretching frequencies in CM^{-1}		Δ frequency in CM^{-1}
	Unbonded	Bonded	Chelated	Unbonded	Bonded	*
		3475		3390 3355	3295	
			3100 to 3000	3360		-500 to -600
			3000 to 2850	3408		-600 to -750

*Difference from unbonded phenol absorption, Table II.

2-Amino-4-nitrophenol and 2-bromoacetamido-4-nitrophenol were measured as KBr pellets, and 2-acetamido-4-nitrophenol in absolute ethanol.

Table II

Literature infrared absorption frequencies

	Compounds	OH stretching frequencies in CM^{-1}			NH stretching frequencies in CM^{-1}		Δ frequency in CM^{-1} *	Ref.
		Unbonded	Bonded	Chelated	Unbonded	Bonded		
Unbonded	ϕ-OH	3610						1
	ϕ-NH_2				3480, 3400			2
	$(\phi)_2$-NH				3430			3
	Trans 2° amides				3460 to 3400			4
Intermolecular Bonds	ϕ-OH + $CH_3-\overset{O}{\overset{\|}{C}}-N(CH_3)_2$		3290				-320	2
	$(\phi)_2$-NH + $CH_3-\overset{O}{\overset{\|}{C}}-N(CH_3)_2$					3320	-110	3
	Trans 2° amides				3320 to 3270			4
Intramolecular Bonds				3220			-390	5
				2960			-650	6

*Differences from unbonded analogues.

References: (1) Kuhn, 1952; (2) Mizushima et al., 1955; (3) Richards and Thompson, 1947; (4) Bellamy, 1958, page 205; (5) Tsuboi, 1952; (6) Hoyer, 1956.

of R_1Br is a similar electron withdrawing group. Intramolecular hydrogen bonds can be formed in seven-membered rings like that of structure I if the ring is stabilized in a planar form. One example of the formation of such a ring is 2-hydroxy-5-terbutyl-2'-hydroxy-3'-chloro-5'-nitroazobenzol. Only a broad band at 3000 cm^{-1} is observed for this compound (Hoyer, 1956). The hydrogen bonded ring in structure I could be similarily stabilized by its partial aromatic character. Intramolecular hydrogen bonded OH stretching frequencies, therefore, can occur around 3000 cm^{-1} where the broad absorption bands for R_1Br and R_1H occur.

For primary and secondary amines the shifts to lower frequencies, upon hydrogen bonding of the NH group, are less than the shifts observed for phenols. Diphenylamine is only shifted from 3430 to 3320 cm^{-1} when it is mixed with N, N-dimethylacetamide in CCl_4 (Richards and Thompson, 1947). The shift is even less for primary amines of which structure II is an example. The NH stretching frequencies of aniline do not change when intermolecular hydrogen bonding with acetone is possible (Mizushima et al., 1955)[3]. Even in compounds where strong intramolecular hydrogen bonds with a carbonyl are indicated by shifts in the carbonyl frequency, the frequency of the NH vibration does not commonly drop below 3300 cm^{-1}. For example, N-methylanthranilate has an absorption peak at 3361 cm^{-1} that can be attributed to the NH stretching frequency (Rasmussen and Brattain, 1949)[4]. Since hydrogen bonded amines have not been demonstrated to absorb at higher frequencies than 3300 cm^{-1}, at least two and probably three absorption bands would be expected to occur

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3 The OH stretching frequency of phenol shifted 210 cm^{-1} to a lower frequency in acetone (Mizushima et al., 1955).

4 In α-amino- α- β -unsaturated ketones the NH absorption frequency is shifted to 3000 cm^{-1}. The lowering of this frequency is attributed to the resonance structure, $R\text{-}C(=O^-)\text{-}C=N^+\text{-}R$ (Cromwell et al., 1949; Flett, 1948). Such a resonance is not possible for structure II.

between 3300 and 3500 cm^{-1} (Bellamy, 1958, p 249) if structure II had been prepared.

One absorption peak in the region of 3300 to 3500 cm^{-1} and a broad band in the region of 3200 to 2900 cm^{-1} is consistent with a trans secondary amide unbonded NH stretching mode (3460 to 3400 cm^{-1}, Bellamy, 1958, p 205), and with an intramolecular hydrogen bonded OH stretching mode, respectively. It is not consistent with a hydrogen bonded primary amine. Therefore, R_1Br can only be 2-bromoacetamido-4-nitrophenol (Structure I). The observed differences in the absorption peaks for R_1H and R_1Br are explained by the electron withdrawing power of bromine in R_1Br.

B. Enzyme Preparations

Reporter labeled α-chymotrypsin The reaction of α-chymotrypsin with 2-bromoacetamido-4-nitrophenol was carried out in a 20% methanol solution with 4 to 8 X 10^{-5} M Worthington bovine α-chymotrypsin and a saturated solution of R_1Br (about 5 x 10^{-4} M) at pH 3 and 20°. The purpose of this reaction was to modify the methionine three residues from the active serine of chymotrypsin with the R_1 group. The desired amount of α-chymotrypsin was dissolved in ice cold 0.001 N HCl in a polyethylene flask, then allowed to come to room temperature. In order to hasten the solution of R_1Br it was dissolved in methanol before addition to the aqueous enzyme solution. At zero time the methanol solution was added slowly to the 20° protein solution and the pH of the solution was adjusted. The flask was covered with aluminum foil to eliminate excess light and was placed in a 20° bath.

A parallel experiment with 2-acetamido-4-nitrophenol in place of R_1Br was run as a control. The loss of activity was followed for 15 to 21 days by the assay method of Schwert and Takenaka (1955) using acetyl-L-tyrosine ethyl ester (see p17). Assays taken during the course of the reaction were measured on reaction solutions which contained the competitive inhibitors R_1Br and R_1H. At the end of the appropriate reaction period, the solution was filtered, then dialyzed for about 5 days at 9° against 3 to 6 baths of 0.001 N HCl that were about 20 times the protein solution volume. Protein preparations were further purified from small molecular weight components on a Sephadex G-25 column (coarse grain). Two hundred to 300 ml of the

dialyzed protein solution were placed on a 3 x 80 cm column at room temperature and eluted with 0.001 N HCl. The protein peaks were followed by 280 mμ absorption and the R_1 peaks by 400 mμ absorption. The protein fractions were quickly frozen for lyophilization. The dialysates from early protein preparations were frozen directly for lyophilization without filtering on Sephadex. The lyophilized protein was stored in the deep freezer.

It was found that R_1Br rapidly cyclizes in neutral or basic solutions to give the colorless product, $\overline{C_6H_3\text{-}(NO_2)\text{-}NH\text{-}CO\text{-}CH_2\text{-}O}$ (M. E. Kirtley and D. E. Koshland, Jr., unpublished). This cyclization was followed by the decrease in absorption at 410 mμ since the cyclic product does not absorb in this region. The half life of the cyclization at pH 7 and room temperature is 15 min. At pH 3 the hydroxyl group of R_1Br is unionized and cyclization does not occur. Modification of α-chymotrypsin at pH 3 by incubation with R_1Br for periods of days was, therefore, possible.

As expected R_1H does not cyclize at neutral pH values. The absorption spectrum of R_1H in phosphate buffer at pH 6.5 does not change when R_1H is kept in this solution for periods of several months.

The absorption at 410 mμ of R_1 modified α- chymotrypsin in phosphate buffer at pH 6.5 decreased with time. This fading is probably due to an intramolecular cyclization of R_1 on the protein with the thioether of the methionine residue acting as the leaving group. The activity of the modified enzyme increased as the fading at 410 mμ occurred indicating that cyclization resulted in a cleavage of R_1 from the enzyme. The half life for this cyclization is about 15 hr. It was, therefore, necessary to store the protein in the dry state or at low pH values. As discussed later, it was also possible to store the modified enzyme in a frozen state at pH 6.5.

Reaction of DFP with α-chymotrypsin Balls, Jansen, and co-workers (1952) found that diisopropyl fluorophosphate (DFP) reacts stoichiometrically with chymotrypsin to yield a phosphoryl protein that is inactive. Schaffer et al. (1953) hydrolyzed the phosphorylchymotrypsin with acid and obtained a serine phosphate. Evidence has accumulated that this serine is at the active site and is essential for the activity of α-chymotrypsin (Strumeyer, White, and Koshland, 1963). To obtain an inert enzyme we, therefore, treated

α-chymotrypsin with DFP. This enzyme was used as a control in the all or none assay (p 16).

To a solution of 4×10^{-4} M α-chymotrypsin was added enough 0.1 M DFP to give a final concentration of 1.09×10^{-3} M DFP. In 0.75 hr no detectable activity by the ATEE assay method was observed. The sensitivity of this assay method for such high protein concentrations was to less than 1% activity relative to the starting enzymatic solution. After 1.08 hr dialysis was begun against 0.001 N HCl that was 40 times the volume of the protein solution. Dialysis was continued for three days with 7 changes of the bath. The protein was then lyophilized and stored in the deep freezer.

C. Enzymatic Assays

Efficiency assay of α-chymotrypsin using acetyl-L- tyrosine ethyl ester This method, adapted after the method of Schwert and Takenaka (1955), was carried out using the Cary 14 recording double beam spectrophotometer equipped with a 0-0.1 optical density slide wire and chart speed of 5 in/min. The assay was conducted by measuring the decrease in absorption at 2370 A as a function of time. The activity was recorded as the slope of the Cary curve at 0.4 min. The rate has the units of Δ absorbance/min/10^{-5} M protein. Alternatively, the activity can be expressed as percent activity relative to the control.

A stock solution of 0.03 M ATEE in isopropanol was stored in the refrigerator. Just prior to use 1 ml of this solution was diluted to 30 ml with 0.05 M phosphate buffer, pH 7.0. Three ml of this solution were pipetted into the reference cuvette and three sample cuvettes.

A blank reading was taken by recording any change in the difference of the absorbance of the sample solutions versus the reference solution. The Cary pen and chart switches were turned off. Five to 100 μ liters of protein solution were pipetted into the sample cuvette. The Cary chart switch was turned on to record zero time. The sample solution was mixed by tipping the cuvette 6 to 10 times. The sample cuvette was replaced in the compartment and the pen switch turned on to measure the assay. The assay slope at 0.4 min was corrected for the slope of the blank reading. The blank reading was usually less than ± 1% of the activity of the unmodified α-chymotrypsin or

about ± 4% of the activity of the R_1-modified α-chymotrypsin.

All-or-none assay for α-chymotrypsin using phenylmethyl-C^{14}-sulfonyl fluoride In this assay chymotrypsin reacted irreversibly with phenyl-methyl-C^{14}-sulfonyl fluoride (PMSF). The percentage of modified enzyme labeled *versus* that of native enzyme indicates the fraction of enzyme retaining at least partial activity (Ray and Koshland, 1963; H. Weiner and D. E. Koshland, Jr., unpublished).

Seventeen mg of enzyme were dissolved in 6.7 ml of 0.05 M phosphate buffer, pH 6.5. Insoluble protein was separated from the solution by centrifuging. At zero time 0.10 ml of PMSF solution (6 mg PMSF/ml dioxane, 0.034 M) was added to the enzyme solution. The final PMSF concentration was 5 times the enzyme concentration. After 1.5 hr at room temperature the protein was separated from the excess PMSF on a Sephadex G-25 column (20 x 2.3 cm) by elution with 0.001 N HCl. Fractions of about 4 ml were collected and analyzed for protein absorption at 280 mμ. The protein peak occurred just after the excluded column volume and was generally spread out over 12 ml. Each fraction was lyophilized to dryness, weighed, and dissolved in a few ml of 0.001 N HCl. The error due to weighing was about 5%. Two mg of each fraction were plated on each of three planchettes. The planchettes were counted at least three times for 8 to 14 min on a Nuclear Chicago windowless gas flow counter.

Samples of the reported-modified α-chymotrypsin before reaction with PMSF and after reaction with PMSF and purification with Sephadex were examined for absorption of the yellow R_1 group at 400 mμ to show that the enzyme was still modified with R_1 after the PMSF treatment.

Corrections for C^{14} self-absorption by the protein during counting were made according to the general methods of Steinberg and Udenfriend (1957). The amount of self absorption is determined by adding increasing amounts of $BaSO_4$ to a fixed amount of C^{14} material. $BaSO_4$ is used since its absorption of β-rays per unit mass is the same as for protein and since it can be accurately weighed (Karnovsky *et al.*, 1955). A plot of the radioactivity of a reference sample per observed radioactivity *versus* total mg of the sample is shown in Figure 5. The axis of the ordinate in this plot represents a correction factor for a particular weight sample. The correction factor for a particular weight protein times the observed count/min/mg at that weight

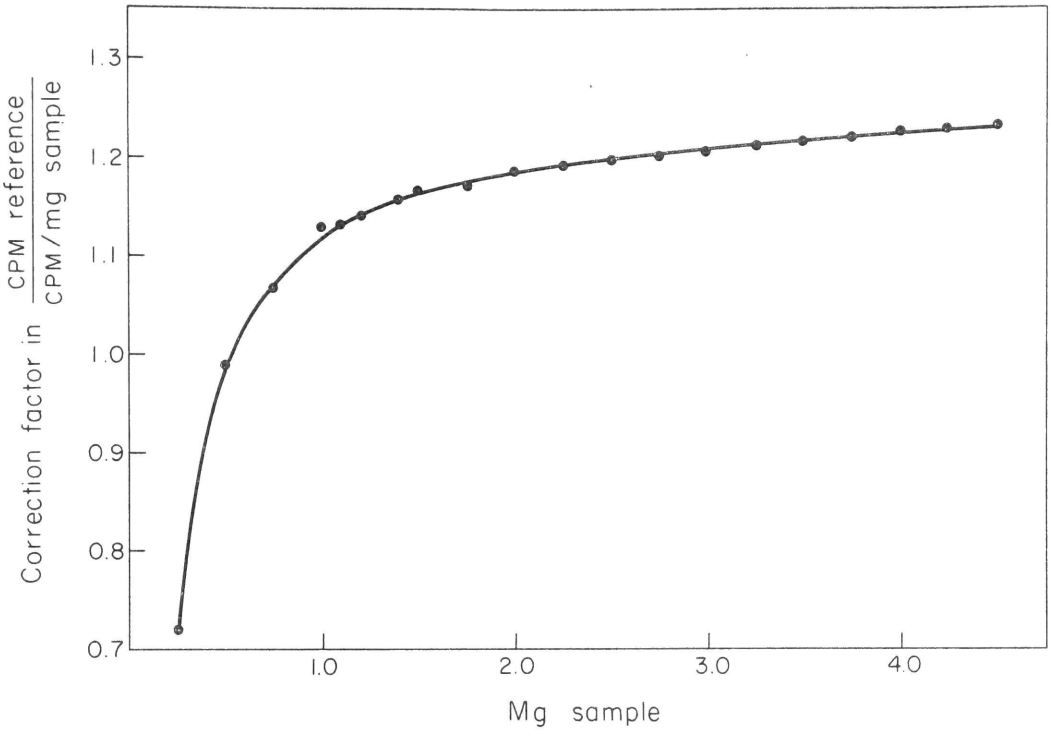

Figure 5 C^{14} Self-absorption curve

The data were obtained from a standard sample of C^{14} with increasing amounts of $BaSO_4$ and were kindly provided by M. E. Koshland and S. M. Gaddone.

gives the corrected count/min/mg.

D. Amino Acid Analyses

Since alkylated methionines are unstable under the conditions normally used for the acid hydrolysis of proteins (Gundlach et al., 1959b), unmodified methionine residues were converted to the acid stable sulfone derivative by the convenient method of performic acid oxidation described by Moore (1963). Subsequent acid hydrolysis and analysis of the number of methionine sulfones gave indirectly the number of modified methionine residues. Cysteine and cystine residues are, also, most accurately determined after performic acid oxidation as cysteic acid (Schram et al., 1954; Moore, 1963).

Performic acid was prepared by mixing 1 ml of 30% H_2O_2 to 9 ml of 97-100% formic acid. After 1 hr at room temperature the performic acid solution was cooled in an ice bath. Two ml of this solution were added to 2 to 5 mg of dry protein in a pyrex ignition tube while in an ice bath. The mixture was allowed to stand overnight at 0°. The excess performic acid was then destroyed by adding 0.3 ml of 48% HBr while swirling the reaction tube in an ice bath. The protein was dried on a Craig rotary evaporator. Twenty ml of 1 N NaOH were added to the condenser to absorb the bromine that distilled over. For hydrolysis 3 ml of 6 N HCl were added. The tubes were then sealed under reduced pressure after the excess air had bubbled free from the solution and were placed in a boiling toluene bath (110°) for 18 hr. Quantitative analysis for amino acid residues was carried out in the routine manner with an automatic recording amino acid analyzer (Spackman et al., 1958), Spinco Division, Beckman Instruments, Inc.

Tyrosine and histidine residues are destroyed by performic acid oxidation. These residues were, therefore, determined by hydrolysis of the protein in 6 N HCl without prior performic acid treatment followed by analysis on the amino acid analyzer. The results are shown in Table VI.

E. Determination of R_1 Residues

The determination of the number of R_1 residues introduced per molecule of protein was accomplished by two different methods.

In the first method a weighed amount of protein was dissolved in 0.5 N NaOH and its absorption measured at 410 mμ and 280 mμ with the Zeiss single beam spectrophotometer. The number of moles of R_1 was calculated from the extinction coefficient for R_1H in 0.5 N NaOH ($\varepsilon = 1.6 \times 10^4$). The amount of protein was calculated from the absorption at 280 mμ in 0.38 N HCl using an extinction coefficient of 5×10^4 (Wu and Laskowski, 1955) after a correction had been made for the absorption of R_1 at 280 mμ (ε, $R_1H = 5 \times 10^3$). Comparison of these values with those determined by weighing the protein showed the presence of approximately 14% inert impurity, probably salt and/or absorbed water.

Since the 410 mμ absorption peak of R_1Br fades in neutral or basic solutions, as discussed on page 14, a second method was devised to determine the number of R_1 residues introduced. In this method the R_1 residues of the modified enzyme were hydrolyzed in 6 N HCl to 2-amino-4-nitrophenol. The moles R_1 per mole protein could be calculated from the absorption of this product at 445 mμ and from the amount of protein as determined by amino acid analysis. Samples of R_1Br, R_1H, and 2-amino-4-nitrophenol were also treated to verify that the product of hydrolysis was 2-amino-4-nitrophenol.

Samples of 1 to 4 mg of colored reagents and of 4 mg of native and modified α-chymotrypsin were weighed to two significant figures and quantitatively transferred to heavy walled test tubes. Two ml 6 N HCl were added to each sample. The samples were evacuated with a vacuum pump for 20 min with alternate freezing and thawing. The hydrolysis tubes were sealed and the samples hydrolyzed at 110° for 24 hr.

The concentration of modified enzyme was determined by amino acid analysis for aspartic acid, glutamic acid, alanine, leucine, and phenylalanine in the hydrolysate. For this determination 0.5 ml of each protein hydrolysate solution was added to a 10 ml beaker. The beakers were covered with watch glasses. The solutions were evaporated to dryness (or in some cases to one-half a drop) in a desiccator over NaOH and silica gel for 3 hr to overnight with a cold water aspirator. The dried hydrolysate was dissolved in 5 ml 0.2 M citrate buffer pH 2.2 for amino acid analysis. The moles of modified protein were calculated using experimentally determined values for the number of each amino acid residue in α-chymotrypsin and for the absorption factor of each

amino acid. Standard Beckman amino acid samples were used for the latter determinations.

To determine the number of R_1 residues the hydrolysates were diluted with NaOH to a solution which was 0.5 N in NaOH and gave a reading of between 0.3 and 0.7 absorbance unit at 445 mμ. The moles R_1 were calculated with the extinction coefficient of 2-amino-4-nitrophenol at its wave length maximum ($\varepsilon = 1.2 \times 10^4$ at 445 mμ). Since the hydrolyzed unmodified α-chymotrypsin was found to absorb at 445 mμ ($\varepsilon = 0.3 \times 10^4$) a correction for protein absorption was made. The extinction coefficients of hydrolyzed R_1Br, R_1H, and 2-amino-4-nitrophenol at 445 mμ ($\varepsilon = 1.36$, 1.41, and 1.26×10^4, respectively) were similar to that for untreated 2-amino-4-nitrophenol. The hydrolysis products also had absorption maxima at 445 mμ, thus verifying that the hydrolysis product was 2-amino-4-nitrophenol.

In order to determine if noncovalently bound reporter groups were absorbed to the enzyme, a sample of the modified enzyme was dissolved in 20% methanol (1mg/1ml), precipitated with 10% trichloroacetic acid (TCA), washed with ether, and dried before it was weighed and hydrolyzed as described. A second sample was dissolved in 20% methanol, precipitated with TCA, then redissolved in 6 M urea and 20% methanol, precipitated, washed and dried before hydrolysis. The number of moles R_1 per mole of methanol-washed-enzyme was compared with that for unwashed enzyme.

F. Spectral Measurements

Spectral measurements were made with the Cary 14 Recording Spectrophotometer equipped with a 0-1.0 or a 0-0.1 optical density slide wire, a scan speed of 25 A/sec, and a chart speed of 5 inches/min. Corrections were made for the air *versus* air absorption of the Cary.

Difference spectra were measured from 700 to 350 mμ with a tungsten lamp and from 350 to 300 mμ with a hydrogen lamp using the 0-0.1 slide wire. Water at 22° was circulated with a Bronwill constant temperature circulating system through cell adapters (144300 Cary Instruments) and the outside of the cell compartments.

Measurement of difference spectra Spectral changes caused by the binding of substrates to reporter modified α-chymotrypsin, CT-(R_1-Met_3)[5], were determined by measurements of difference spectra.

Large batches of protein were prepared for these measurements in order to minimize variations due to the fading of the enzyme-bonded reporter group spectrum near 400 mμ with time as discussed on page 14. Since the half life of this fading phenomenon is about 15 hr at pH 6.5 at room temperature, measurements made within 1 hr of each other differed by less than 10%. It was also observed that CT-(R_1-Met_3) in 0.05 M phosphate could be indefinitely frozen without effecting the magnitude and character of the enzyme spectrum or its difference spectra with substrates. A large batch of homogenous protein solution could, therefore, be prepared and frozen. Since about 10% of the R_1Br treated enzyme was insoluable at pH 6.5, the solution was centrifuged before freezing. Small quantities of the protein could be unfrozen just prior to use so that quantitative comparisons of substrate or pH-generated spectral changes could be made.

Large batches of enzyme were prepared as follows. One hundred to two hundred mg were dissolved in ice cold 0.05 M phosphate buffer, pH 6.5, at a concentration of 2 mg/ml. After 15 min the solution was placed in a 22° bath and the pH was adjusted to 6.50 with 1 N NaOH. The solution was in the 22° bath or at room temperature for 30 min before it was returned to an ice bath. In order to remove the undissolved protein, the solution was centrifuged at 12,000 rpm and 0° for 30 min. Samples of 6 to 10 ml protein solution were then poured into polyethylene test tubes (polyethylene was used so that there would be no absorption of the protein to the test tube walls) and about 2 hr after the initial solution of the protein, the samples were frozen in a dry ice and acetone bath. The solution was, therefore, 30 min at 22° and 90 min at 0° before being frozen and stored in the deep freezer.

In preparation for the difference spectra, substrate was dissolved in 0.05 M phosphate buffer and adjusted to pH 6.50. Then the 6 to 10 ml enzyme

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5 The nomenclature, CT-(R_1-Met_3), reflects the attachment of the reporter group, R_1, to the methionine three residues from the active serine of chymotrypsin.

samples were unfrozen by placing them in a 22° bath for 15 min. During this time 1 ml of substrate solution was pipetted into the substrate side of two split-compartment quartz cuvettes (Figure 6; Yankeelov, 1963). Several cuvettes were filled if time changes were to be followed. The same 1 ml pipette was used for all deliveries to the cuvettes.

After the protein solution was unfrozen at 22°, the 1 ml pipette was rinsed 3 times with the protein solution to eliminate variations due to absorption of the protein to glass. Each rinse was discarded. One ml of protein was then delivered to the enzyme compartment of each split-compartment cuvette. The cuvettes were covered with parafilm.

A blank difference spectrum of the sample cuvette versus the reference cuvette was measured in order to assure that no pH changes had thus far occurred and that the two cuvettes contained optically identical solutions.

After the blank spectrum measurement the sample cuvette was mixed by tipping 20 times. The difference spectrum was measured from 30 to 60 min after the frozen enzyme solution had been in a 22° bath. During this half-hour period the variations of the spectra due to the fading of the protein were less than 5%.

At the end of the experiment the pH of the protein and substrate solutions were measured to ascertain that there were no differences greater than 0.01 pH unit. Measurements could be made in the split-compartment cuvettes with Leeds and Northrop miniature glass and reference electrodes (No. 124138).

Errors due to pH and ionic strength variations and pipetting To determine the magnitude and character of a difference spectrum due to a pH shift, a difference spectrum was measured in the manner described except that 0.05 M phosphate buffer at different pH values was added instead of substrate[6] (Figure 7). A maximal difference of 0.002 unit of absorbance was observed for a pH difference of 0.01 unit around pH 6.5 The maximum occurred

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6 In the early work the method used for measuring difference spectra differed from that described in the following ways: Solutions of enzymes were

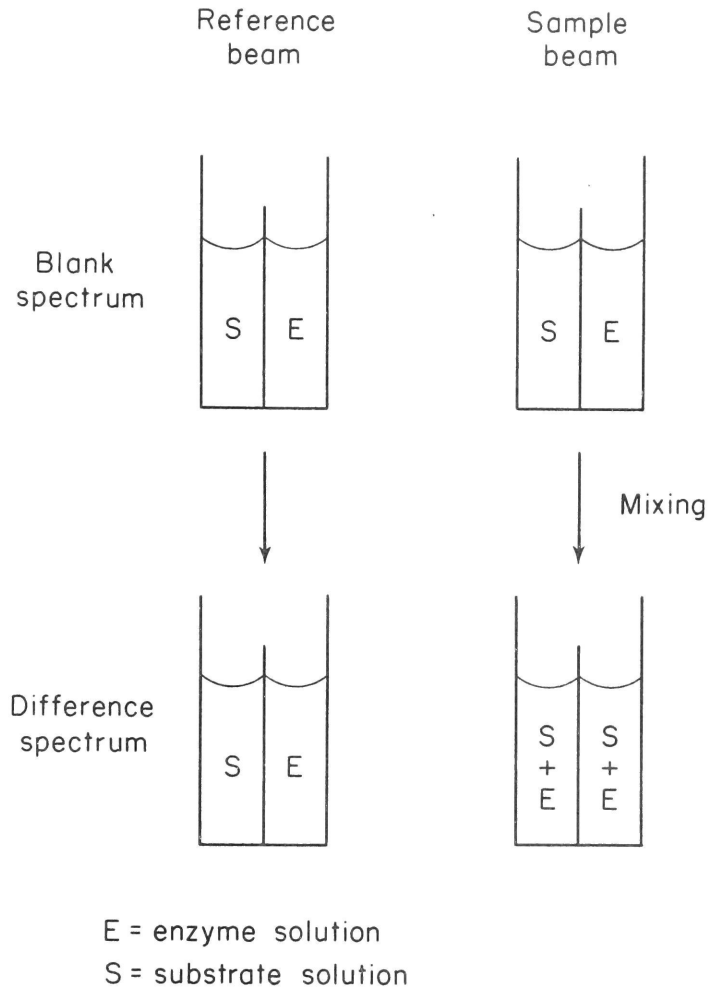

Figure 6 Method for measuring difference spectra

The split-compartment quartz cuvettes have a total path length of 0.880 cm. One ml substrate solution (S) and one ml enzyme solution are added to each cuvette. After a blank spectral reading the sample solution is mixed and the difference spectrum is read.

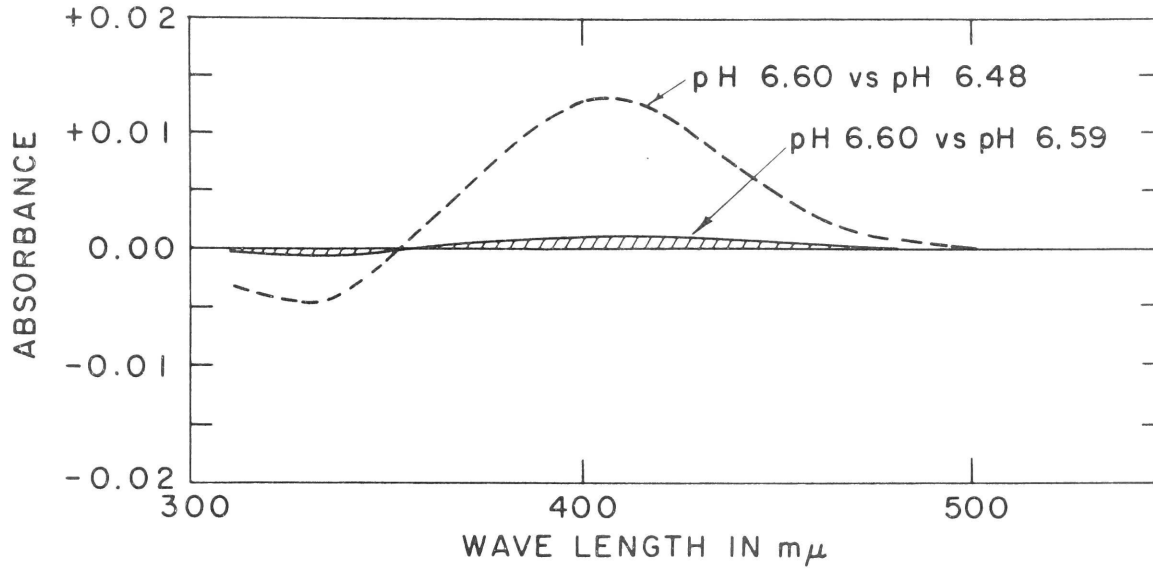

Figure 7 Difference spectrum of CT-(R_1-Met_3) generated by a change in pH

Conditions[6]: 7.4×10^{-5} M enzyme, 0.05 M phosphate buffer. The hatched-in area represents a pH change of 0.01 pH unit, calculated from the measured spectrum for a change of 0.12 pH unit.

at about 405 mμ. There was an intersection with the base line at about 350 mμ and a minimum at about 330 mμ.

To determine the effect of an ionic strength change, acetic acid which does not bind to chymotrypsin was added instead of substrate to the enzyme solution[6] (Figure 8). The difference observed for 7.4×10^{-5} M enzyme and 1.5×10^{-3} M acetic acid in 0.05 M phosphate buffer was less than 0.001 unit of absorbance. The difference observed for 6.5×10^{-5} M enzyme and 16.8×10^{-3} M acetic acid (a change in ionic strength of about 10%) was 0.0025 unit of absorbance at 480 mμ and less than 0.0015 unit from 430 to 300 mμ.

The magnitude and character of the difference spectra arising from errors in pipetting were determined by adding 5 and 25 μ liters of phosphate buffer to the 2 ml protein solution in the sample cuvette and none to the protein reference solution[6]. A maximum difference of 0.001 and 0.005 unit of absorbance were observed at 390 mμ for an error of 5 and 25 μ liters, respectively. The errors due to pH variation, ionic strength change, and pipetting are less than 0.003 unit of an absorbance as determined by the above experiments.

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dissolved and centrifuged on the day of use; The difference spectra were measured from about 2 hr to 8 hr after the enzyme was dissolved; No cell adapter was used for temperature control so that differences in temperature between various experiments as well as differences in temperature between the reference and sample cuvettes could have occurred; Two ml of the protein solution and 0.1 to 0.5 ml of the substrate or buffer solution were added to three-milliliter, single compartment, quartz cuvettes; Substrate absorptions were determined separately and subtracted from the difference spectra. Though the difference spectra determined by this method were qualitatively the same as those determined by the improved method, the improved method is recommended for quantitative comparisons.

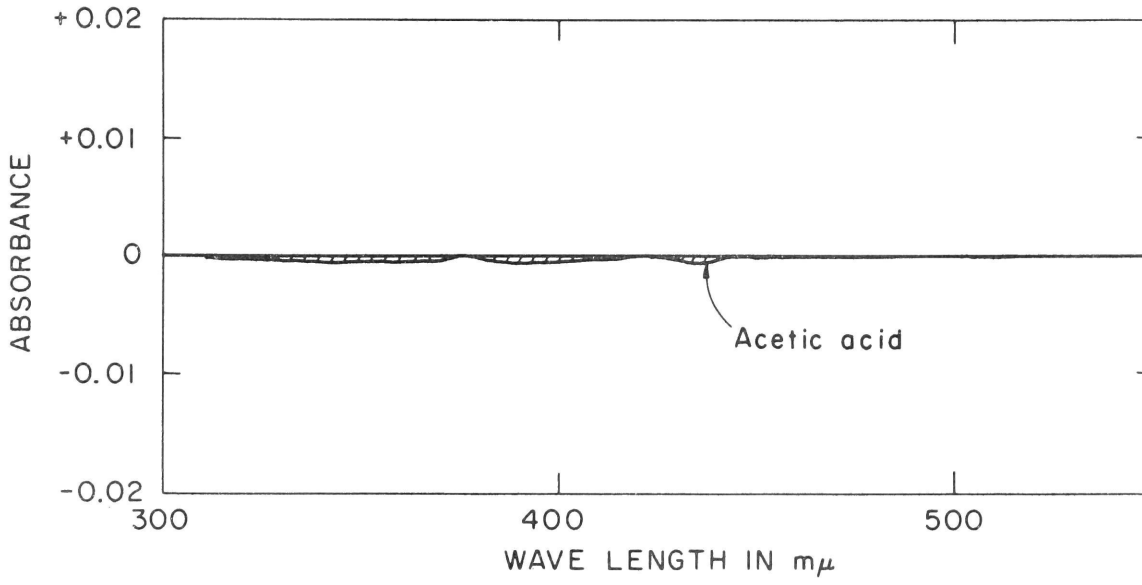

Figure 8 Difference spectrum of CT-(R_1- Met_3) with acetic acid

Conditions[6]: 7.4×10^{-5} M enzyme, 1.5×10^{-3} M acetic acid, 0.05 M phosphate buffer, pH 6.49 ± 0.01.

Non-covalently bound reporter groups To establish that the substrate-generated difference spectra only occur when the reporter group is covalently bonded to the enzyme at the concentration of R_1 less than or equal to the enzyme concentration, difference spectra of R_1H or R_1H and protein with and without substrates were measured. These difference spectra were measured as described[6], except that solutions of R_1H or R_1H and α-chymotrypsin replaced the solution of CT-(R_1-Met_3). The data in Table III show that for substrate concentrations from 1.5 to 17. x 10^{-3} M the maximum absorbance difference is usually less than 0.004 unit. All measurements were made before the cuvette solutions were thermostated, and, therefore, the differences probably represent thermal variations.

Table III

Substrate-induced spectral changes of R_1H and R_1H + α-chymotrypsin

Substrates Added	Concentration of Substrates in 10^{-3} M	Maximum Absorbance Difference
acetyl-L-phenylalanine	17.	0.001 at 460 mμ
benzoyl-L-alanine	2.3	0.003 at 447 mμ
benzoyl-D-alanine	2.3	0.004 at 430 mμ
benzoyl-D, L-alanine*	12.	-0.004 at 395 mμ
" " **	15.	-0.005 at 395 mμ
benzoyl-L-phenylalanine	6.4	$\leq$0.001
benzoyl-D-phenylalanine	2.0	0.004 at 435 mμ
" "	1.5	0.004 at 325 mμ
benzoyl-D, L-phenylalanine*	4.0	$\leq$0.001
" " **	4.9	-0.004 at 400 mμ

Conditions: 3.5×10^{-5} M R_1H; 7×10^{-5} M α-chymotrypsin except where indicated by *: 3.5×10^{-5} M α - chymotrypsin and by **: no protein.

Section III

Results

RESULTS

A. Characterization of the Reporter-Labeled Protein

Activity of the enzyme by the efficiency assay method The loss of the activity of α-chymotrypsin during its reaction with R_1Br was determined by the efficiency assay method described using acetyltyrosine ethyl ester (ATEE) as the substrate. The control treated with R_1H lost about 10% activity in 15 to 20 days. The enzymatic activity of the R_1Br-treated chymotrypsin relative to the chymotrypsin control leveled off in 15 days and remained about 46% (Figure 9).

The enzymatic activity in 12 days was about 50% or within 4% that of the 15 to 20 day old reaction mixture. The enzyme preparations used in the following studies were incubated 12 and 18 days then dialyzed to remove excess R_1Br. Therefore, they should have had little variation in the amount of reporter group bonded.

It was difficult to determine the activity of the modified protein by the ATEE efficiency assay due to the presence of about 30% unmodified α-chymotrypsin as described later. This assay indicates, however, that the efficiency of the modified enzyme is less than 30% that of the native enzyme.

Activity of the enzyme by the "all-or-none" assay method The "all-or-none" assay method was used to deterimine if all of the reporter-labeled enzyme molecules retained some activity or if some of these molecules had no activity. In this assay substrate is irreversibly bonded to the active site so that all species with any activity react. An enzyme that has a reduced binding constant, for instance, would register as 100% active by this method but would have a reduced activity in an efficiency assay such as the ATEE assay.

In the all-or-none assay developed by Drs. H. Weiner and D. E. Koshland, Jr. (unpublished), chymotrypsin preparations are allowed to react with phenylmethyl-C^{14}-sulfonyl fluoride (PMSF) until all the enzyme molecules with any activity are reacted. Since the rate of cleavage of phenylmethylsulfonyl from chymotrypsin is negligible below 25° between pH 2 and 9 (Gold and Fahrney, 1964), only inert enzyme would fail to be labeled. The results

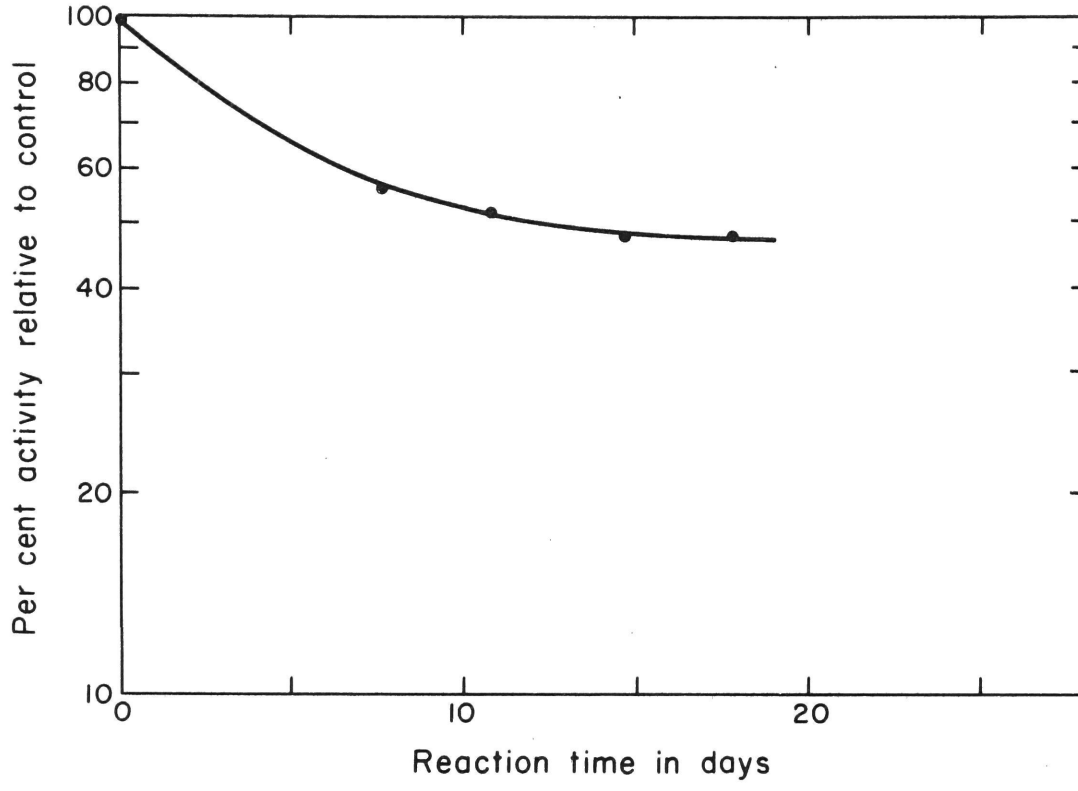

Figure 9 Reaction of α-chymotrypsin with R_1Br

Effect of reporter group reagent on the activity of α-chymotrypsin relative to an enzyme control as measured by the efficiency assay using ATEE. Conditions of the reaction: 8×10^{-5} M α-chymotrypsin, 20 percent methanol, 5×10^{-4} M R_1Br (saturated), pH 3, 20°.

from this reaction are given in Table IV. The incorporation of C^{14} into the native α-chymotrypsin is 3,920 count/min. Reporter-modified α-chymotrypsin has essentially 100% activity. Chymotrypsin treated with diisopropyl fluorophosphate (DFP) has less than 1% activity by this method, indicating that PMSF must react specifically with the active site rather than nonspecifically with the protein. The reporter-modified enzyme treated with PMSF and filtered on Sephadex G-25 was yellow in a basic, pH 13 solution and had an absorption at 400 mμ indicating that the modified enzyme had not reverted to unmodified α-chymotrypsin during the PMSF treatment at pH 6.5. The results from this all-or-none assay show that all the reporter-modified enzyme molecules are active, even though some alteration of K_m or V_m occurred, as shown by the ATEE assay.

Amino acid residues modified by reaction with R_1Br Analyses of the acid hydrolysates of α-chymotrypsin and reporter-modified enzyme oxidized with performic acid showed that about 0.7 to 0.8 mole of methionine residues is modified by treatment with R_1Br for 18 days (Table V and VI). No other amino acids are detectably modified as determined by the analyses of these hydrolysates and of acid hydrolysates which had not been treated with performic acid (Table VI). No analyses for tryptophan residues were performed, since studies with iodoacetic acid showed that tryptophan residues are not modified (Gundlach et al., 1962a; Koshland et al., 1962a).

The position of the reporter-modified methionine residue has not yet been unequivocally established. Because previous modification studies of methionine showed that only the methionine three residues from the active serine reacts in aqueous solutions, (Koshland et al., 1962a) it is likely that R_1Br modified this residue as well.

Number of R_1 residues introduced per enzyme molecule It was important to know the number of R_1 residues per enzyme molecule in order to verify that no residues other than the methionine residues are modified by R_1Br and that no R_1 residues are noncovalently bound to the enzyme.

The moles of R_1 residues per mole of α-chymotrypsin were determined from the 410 mμ absorption of a basic solution of the reporter-modified enzyme to be 0.5 to 0.7 (Table V). By the more accurate method of acid hydrolysis and spectrophotometric analysis of the hydrolysate at 445 mμ

Table IV

Phenylmethyl-C^{14}-sulfonyl fluoride "all-or-none" assay

Protein	Amount of Radioactivity on Protein in cpm/mg	All-or-None Activity Relative to Native Protein
Native α -CT	3,920	100.
CT-(R_1-Met_3)	3,710	94.6
α-CT, DFP treated	24	0.6

All values were corrected for C^{14} protein self absorption.

Conditions: 8×10^{-5} M protein, 5×10^{-4} M phenylmethyl-C^{14}-sulfonyl fluoride, pH 6.5, 24^{o} for 1.5 hr.

Table V

Determination of R_1 residues per molecule of α-chymotrypsin

Residues Modified or Introduced	Number of Residues in Native α-CT	Number of Residues After Reaction of α-CT with R_1Br
Methionine sulfone[(a)]	2.0	1.2
Methionines modified		0.8
R_1 Residues[(b)]		0.6
R_1 Residues[(c)]		0.5 - 0.7

(a) The number of unmodified methionine residues was determined as methionine sulfone after performic acid oxidation. The average number of methionine sulfone residues for native α-chymotrypsin was 2.16. Methionine sulfone residues were normalized to 2.0 residues per molecule of α-chymotrypsin.

(b) The number of residues was determined after acid hydrolysis, as 2-amino-4-nitrophenol by absorption at 445 mμ, pH 12.

(c) The number of residues was determined by spectrophotometric analysis of the protein in a basic solution using the extinction coefficient for R_1H at 410 mμ, pH 12.

Table VI

Amino acid residues modified by R_1Br treatment of α-chymotrypsin

Amino Acid Residue	Literature Value (1)	Performic Acid Treated α-CT Control (2)	Performic Acid Treated CT-(R_1-Met_3) (3)
Lysine	14	14.9*	14.3
Histidine	2		2.0 (4)
Arginine	3	3.0*	3.0
Cysteic acid	10	9.7*	9.7
Aspartic acid	22	24.5	24.5
Methionine sulfone	2	2.16	1.33
Threonine	22	20.9	20.6
Serine	28	22.0	21.3
Glutamic acid	15	15.0	15.9
Proline	9	9.3	9.7
Glycine	23	23.8	24.0
Alanine	22	22.9	23.1
Valine	23	23.2	23.7
Isoleucine	10	9.8	9.8
Leucine	19	19.	19.
Tyrosine	4	4.0 (4)	4.1 (4)
Phenylalanine	6	5.7	5.6

Performic acid hydrolysis and acid hydrolysis were carried out according to the procedure of Moore, 1963. The number of leucine residues were assumed to be 19 in each determination.

(1) Values from Hartley's sequence determination, 1964b.
(2) Conditions the same as for (3) except without R_1Br. The values are from an average of 3 determinations except when indicated by*: from an average of two determinations. The average error was ≤ 0.3 residues.
(3) Conditions: 8×10^{-5} M α-chymotrypsin, 5×10^{-4} M R_1Br (saturated), 20 percent methanol, pH 3, and 20° for 18 days. Values from one determination.
(4) Determined directly by acid hydrolysis without performic acid treatment.

the moles of R_1 per mole of enzyme were found to be 0.6. When a sample of the modified enzyme was purified with methanol or methanol and urea, the moles of R_1 residues per mole of chymotrypsin did not significantly differ (0.6 to 0.5 mole), which indicates that R_1 was covalently bonded to chymotrypsin.

The number of R_1 residues introduced per molecule of enzyme agrees well with the number of methionine residues modified (Table V), and no other amino acid residues were detectably modified. Methionine is, therefore, the only amino acid residue of α-chymotrypsin modified by R_1Br under these conditions, and no significant number of R_1 residues is noncovalently bound.

Visible absorption spectra The spectra of the reporter group (R_1H), of unmodified α-chymotrypsin, and of reporter-labeled chymotrypsin [(CT-)R_1-Met_3)], are given in Figure 10. All of the spectra were taken in 0.05 M phosphate buffer at pH 6.5.

R_1H has a major peak in the visible region with a maximum between 405 and 410 mμ, a second smaller peak at 310 mμ, and a peak in the ultra-violet region at about 285 mμ. Titration studies to be reported in a later section show that the 410 and 310 mμ peaks are due to the absorption of the unionized and ionized form of the phenol, respectively (Figure 17).

α-Chymotrypsin has no significant absorption above 315 mμ. Its major peak occurs at 278 mμ.

When R_1 is covalently attached to α-chymotrypsin, its absorption peak at pH 6.5 due to the ionized phenolic species is shifted about 12 mμ in the direction of shorter wave lengths. The exact position of the maximum of the peak due to the unionized form of R_1 is obscured by the protein absorption peak.

B. Measurements of Difference Spectra

Interpretation of difference spectra Difference spectra can be caused by changes in intensity (extinction coefficient at a peak of absorption), shifts in the position of the maximum of the peak, and broadening of the peak. A change in the intensity of the absorption peak is shown in Figure 11a. The spectra of the reference and sample solutions are labeled R and S, respectively. The maximum of the difference spectrum (D) falls at the position of the two solution absorption maxima. If the sample solution

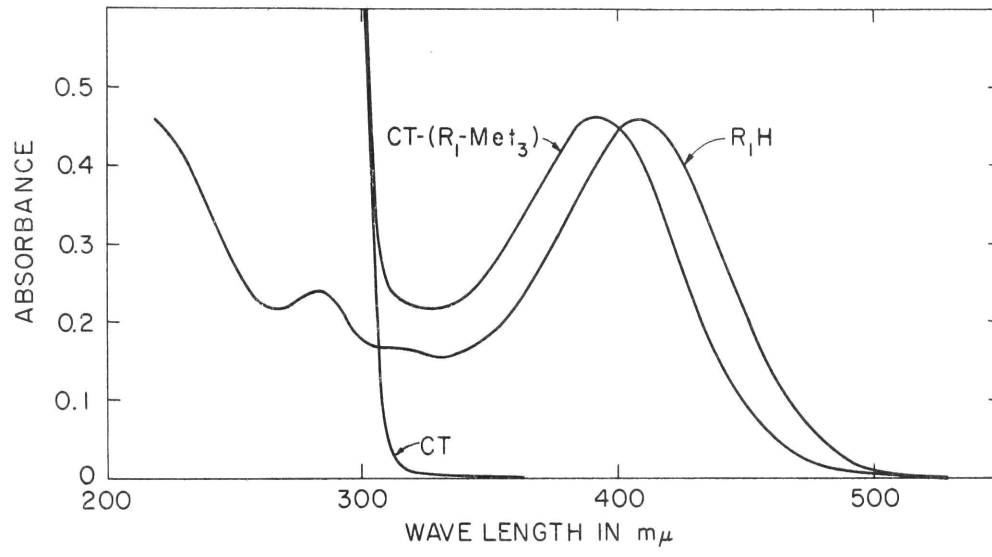

Figure 10 Influence of α-chymotrypsin environment on reporter group spectrum

Spectra of chymotrypsin (CT), 2-acetamido-4-nitrophenol (R_1H), and chymotrypsin which has been reacted with 2-bromoacetamido--4-nitrophenol. Conditions[6]: 7.4×10^{-5} M CT-(R_1-Met_3) or CT, or 4.05×10^{-5} M R_1 H in 0.05 M phosphate buffer, pH 6.49.

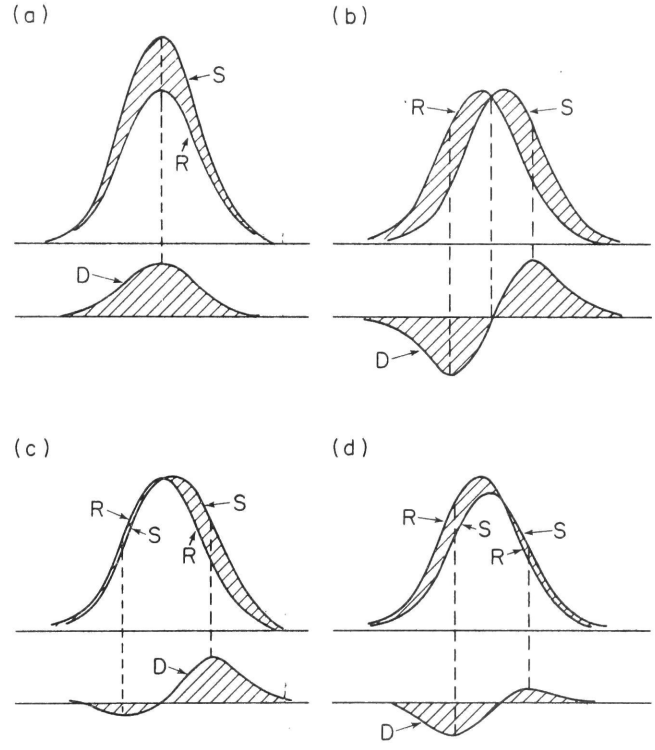

Figure 11 Origin of difference spectra

The difference spectra (D) represent a change in the spectra of the sample solutions (S) relative to the reference solution (R) by (a) an increase in intensity, (b) a shift to a longer wave length, (c) a broadening of the spectrum and a slight shift to a longer wave length, and (d) both a decrease in intensity and a shift to a longer wave length.

increases in intensity, as in this drawing, the difference spectrum lies above the base line; if it decreases in intensity the difference spectrum lies below the base line.

In Figure 11b the spectrum of the sample solution is shifted to a longer wave length relative to the reference solution spectrum. The areas of the difference spectrum above and below the base line are equal. The maximum and minimum of the difference spectrum fall between the inflection points of the two solution spectra. The intersection of the difference spectrum with the base line is at the point of intersection of the two solution spectra.

The absorption peak of the sample solution is broader than the reference solution in Figure 11c. Its absorption maximum is also shifted to a slightly longer wave length. The maximum of the difference spectrum occurs between the inflection points of the absorption spectra of the reference and sample solutions. The intersection with the base line is near the position of the maxima of the two solution absorption spectra. The position of the maximum in the difference spectra and the intersection with the base line can often be used to distinguish between spectral broadening and changes in intensity.

In Figure 11d both a change in the intensity of the absorption peak and a shift of the peak maximum to a longer wave length are shown. The area below the base line is greater than that above due to the decrease in intensity of the solution absorption.

Substrate-generated spectral changes of reporter-labeled α-chymotrypsin To measure the spectral changes due to the addition of substrates, amino acid derivatives such as benzoyl-L-phenylalanine were used. The carboxylate ion form of the acid which is the substrate for the reverse reaction, was chosen in order to avoid pH changes due to the hydrolysis of the substrates. The pH change could be controlled for amides but it was also desirable at this point to exclude changes due to the various catalytic steps. Since the rate of the reverse reaction is negligible, the differences we measured were only due to the binding of the substrate to the active site. The original data for the difference spectrum generated by benzoyl-L-phenylalanine (1×10^{-2} M) is shown in Figure 12. The shape of

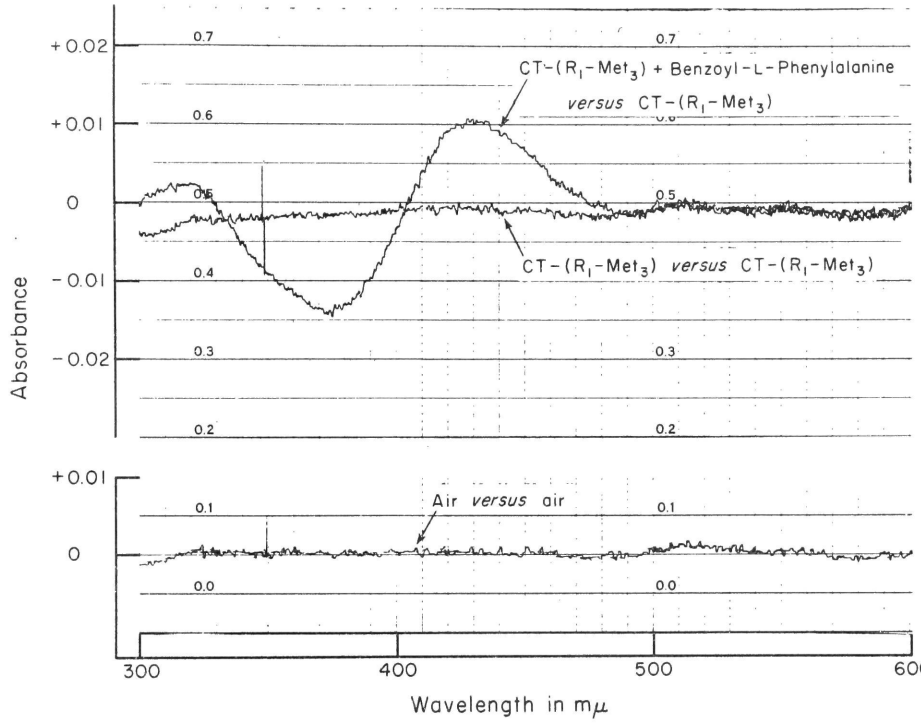

Figure 12 Substrate-generated difference spectrum of chymotrypsin labeled with reporter group: Original Data

Conditions: 2.2×10^{-5} M CT-(R_1-Met_3), 1×10^{-2} M benzol-L--phenylalanine, 0.05 M phosphate buffer, pH 6.51 ± 0.01, path length 0.88 cm.

the blank spectrum, CT-(R_1-Met_3) *versus* CT-(R_1-Met_3) differs from that of the air *versus* air spectrum by only ± 0.001 absorbance unit. The difference spectra are redrawn as the difference between the blank spectra and the substrate-generated difference spectra. A drawing of this difference spectrum appears in Figure 13.

Before discussing the difference spectra observed when substrates are added to the reporter-labeled enzyme, the size and character of the difference spectra due to changes in pH and ionic strength are reported in order to rule out changes in these properties as contributors to the substrate-generated spectra. A difference spectrum generated by a change in pH has a peak that extends from 500 to 350 mμ with a maximum around 400 mμ (Figure 7). Since the maximum of the ionized phenolic peak occurs near 400 mμ, this observed difference represents mainly an increase in intensity when the pH of the solution is increased. There is also a small decrease in intensity around 320 mμ which is due to a change in the unionized phenolic peak. When the pH is controlled to within 0.01 pH unit as it was for the substrate-generated difference spectra, the observed spectral change due to pH variation is less than 0.002 absorbance unit (Figures 7 and 14e). This difference is small compared to the difference spectra generated by benzoyl-L- and D-phenylalanine and phenylpropionic acid that are shown in Figure 14 a, b, and c.

Figures 8 and 14d show that the addition of an anion that does not bind to α-chymotrypsin but changes the ionic strength causes a difference spectrum of less than 0.001 of an absorbance unit. A comparison of the difference spectrum afterthe addition of acetic acid with those after the addition of the same concentration of substrates (Figure 14) shows that the substrate-generated spectra cannot be due to an ionic strength change of the medium.

The difference spectra generated by the addition of benzoyl-L- and D-phenylalanine and phenylypropionic acid have different characters (Figure 14). The interpretations of these substrate-generated difference spectra were facilitated by drawing the spectra of the enzyme-substrate mixtures. An example of this method of analysis is given in Figure 13 for an experiment with benzoyl-L-phenylalanine at a higher concentration than that shown in Figure 14. The line for the substrate-generated spectrum was drawn from the difference spectrum for 1×10^{-2} M benzoyl-L-phenylalanine

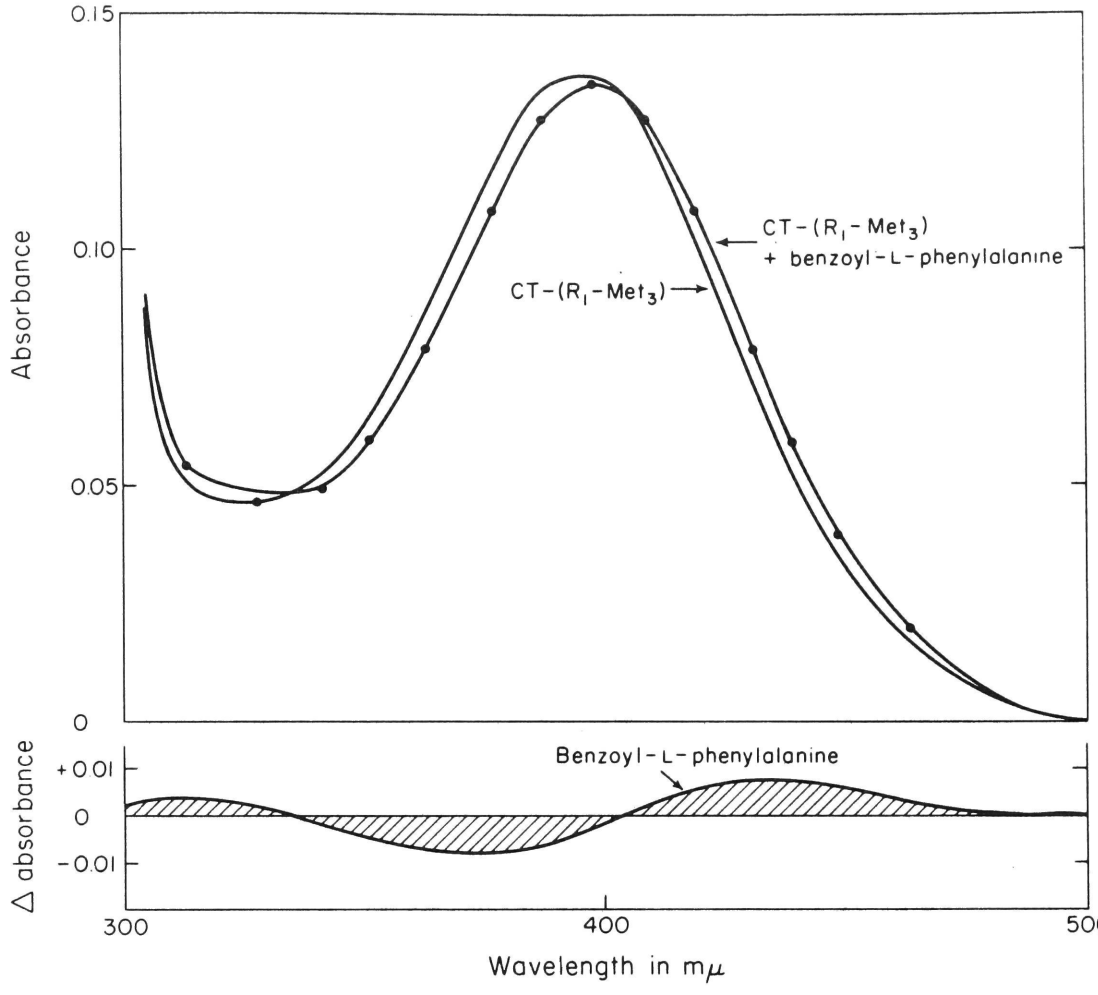

Figure 13 Substrate-generated difference spectrum and its solution spectra

The spectrum of the enzyme-substrate sample solution, CT-(R_1-Met_3) with benzoyl-L-phenylalanine, was drawn from the sum of the measured enzyme reference solution spectrum, CT-(R_1-Met_3), and the measured difference spectrum (hatched area). The points which nearly coincide with the observed sample solution spectrum were calculated by assuming a 2 percent decrease in intensity of the reference solution spectrum and a shift of 3 mμ to a longer wave length. Conditions: 2.2×10^{-5} M CT-(R_1-Met_3), 1×10^{-2} M benzoyl-L-phenylalanine, 0.05 M phosphate buffer, pH 6.51 ± 0.01, path length 0.88 cm.

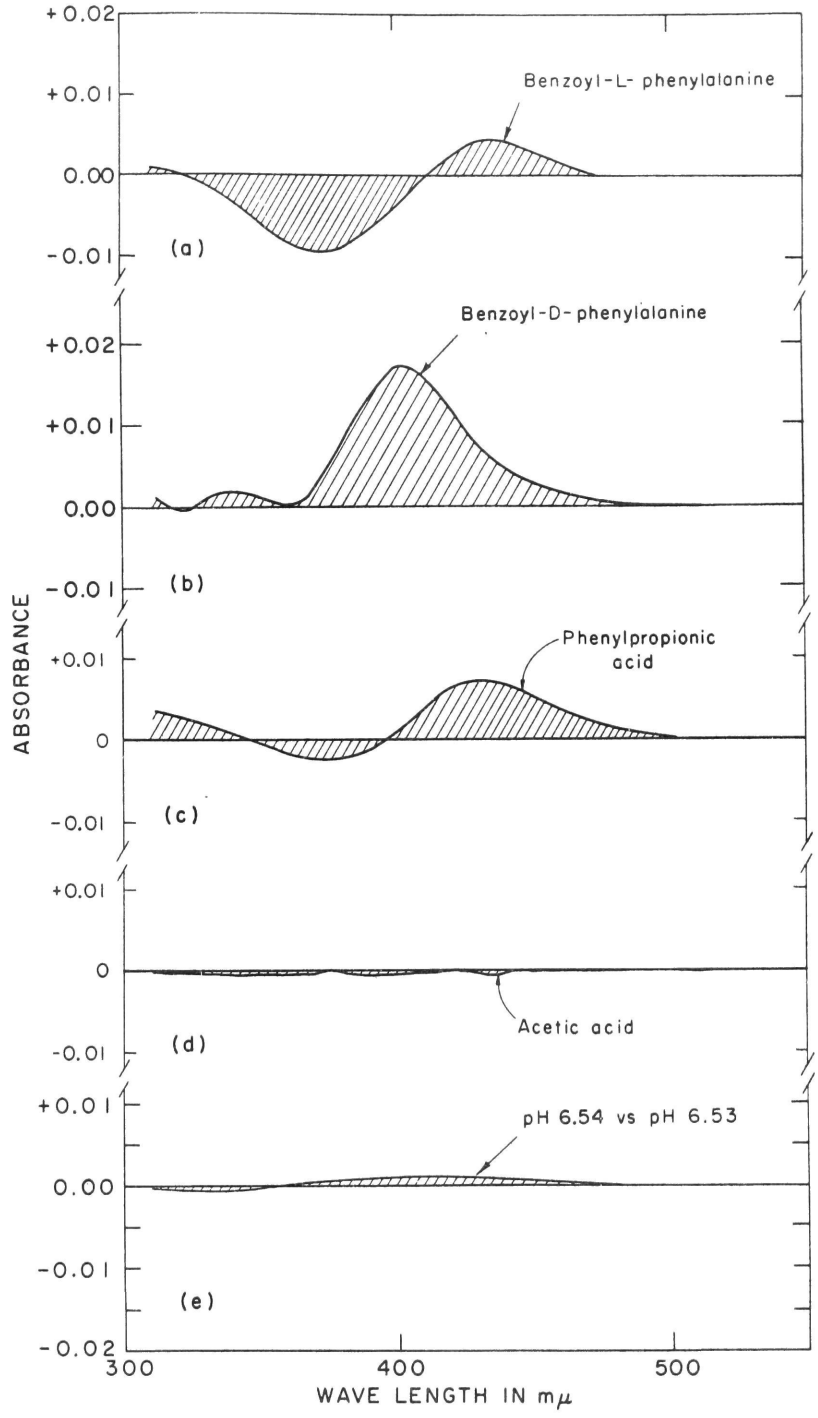

Figure 14 Substrate-generated difference spectra and nonsubstrate controls

Difference spectra of chymotrypsin labeled with a reporter group, CT-(R_1-Met_3). Conditions[6]: 7.4×10^{-5} M enzyme, 0.05 M phosphate buffer; (a)-(d) 1.5×10^{-3} M substrate, pH 6.49±0.01; (e) spectrum calculated for pH change of 0.01 pH units, based on measured spectra for a change of 0.10 and 0.02 pH units.

which is given at the bottom of the figure and the measured spectrum of the reference solution [2.2 x 10^{-5}M CT-(R_1-Met_3)]. This drawing shows that the spectral perturbation involves both a decrease in intensity and a shift to longer wave lengths. The points were calculated assuming that the decrease in intensity was 2% and the wave length shift was + 3 mμ. The points differ from the observed spectral change only in the neighborhood of the unionized absorption peak (315 mμ). If a calculation were made for an increase in the absorption of the 315 mμ peak the points in this region would probably coincide with the observed spectral change.

The spectrum of the enzyme-substrate mixture which is drawn is the sum of two discrete absorption peaks, that for the reporter-modified enzyme-substrate complex and that for the absorption for the reporter-modified enzyme. The absorption due to the reporter-modified enzyme-substrate complex represents only a small fraction of the total absorption of the spectrum since the concentration of substrate is well below that required for saturation of the enzyme. Attempts to saturate the enzyme with substrates were unsuccessful due to the low solubility of the aromatic substrates in aqueous solutions.

Spectral changes due to the addition of various substrates can now be analyzed and compared. The spectral perturbation due to the addition of 1.5 x 10^{-3} M benzoyl-L-phenylalanine to 7.4 x 10^{-5} M CT-(R_1-Met) represents both a shift to a longer wave length (+ 1 mμ) and a decrease in intensity (1.3%). Slight variations in the character of this spectrum with that shown in Figure 13 are probably due to the differences in the treatment of the enzyme before the spectral measurements were measured, especially in terms of the time the enzyme was at neutral pH values at room temperature. A comparison of these two spectra, however, does show that saturation of the enzyme with substrate has not been reached.

The spectral perturbation due to the addition of benzoyl-D-phenylalanine has a very different character from that of the L isomer. The difference maximum occurs near the position of the R_1 absorption peak (400 mμ), indicating that the spectrum represents mainly an increase in the intensity of absorption (about 3%) upon the addition of substrate. There is only a slight shift (about + 1 mμ) in the wave length maximum. The observation that the D isomer of benzoylphenylalanine causes an increase in intensity and the L isomer a decrease in intensity suggests that the isomers do not bind in the

same manner at the active site.

The perturbation due to the additon of phenylpropionic acid (Figure 14c) is not as great as that due to the addition of the benzoyl-phenylalanines. It represents a shift of about 1 mμ to a longer wave length and a slight broadening of the spectrum.

Since benzoylalanine and acetylphenylalanine have one less aromatic group than benzoylphenylalanine, it was of interest to compare the character of the perturbations they produced. These two substrates might also be used to distinguish between interactions with the active site by large substituents at the amine position of the amino acid (benzoylalanine) and at the side-chain position of the amino acid (acetylphenylalanine).

Figures 15 a-d are the difference spectra due to the addition of 1.5×10^{-3} M benzoyl-L- and D-alanine and acetyl-L- and D-phenylalanine. Though the concentrations of the enzyme and substrates are the same as those for Figure 12, the perturbation is less (0.005 to 0.02 unit of absorbance in Figure 14 and less than 0.003 unit in Figure 15). Much of this decrease in absorption may be accounted for by a lower binding affinity of these substrates to the enzyme than that for benzoylphenylalanine. A comparison of the K_m values for acetyl-L-phenylalanine amide (31 mM, Foster and Niemann, 1955a) and benzoyl-L-phenylalanine amide (3.5 mM, Hein and Niemann, 1961) to chymotrypsin indicates that this interpretation may be correct[7].

At concentrations of substrate 30 times those for Figure 15 at half the enzyme concentration, appreciable difference spectra resulted (Figure 16). Some of the variations in the characters of these spectra with those from Figure 15 may be due to the errors in measuring small differences such as those in Figure 15. All the difference spectra in Figure 16 represent shifts of the ionized absorption peak to longer wave lengths. That for

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7 The true binding constant, K_m, of chymotrypsin closely approximates the measured or apparent binding constant, K_m (app), for amides since the acylation step is rate controling. For esters K_m (app) is smaller than the true binding constant since deacylation is rate limiting (Zerner and Bender, 1963). Whenever available we have used the values for amides.

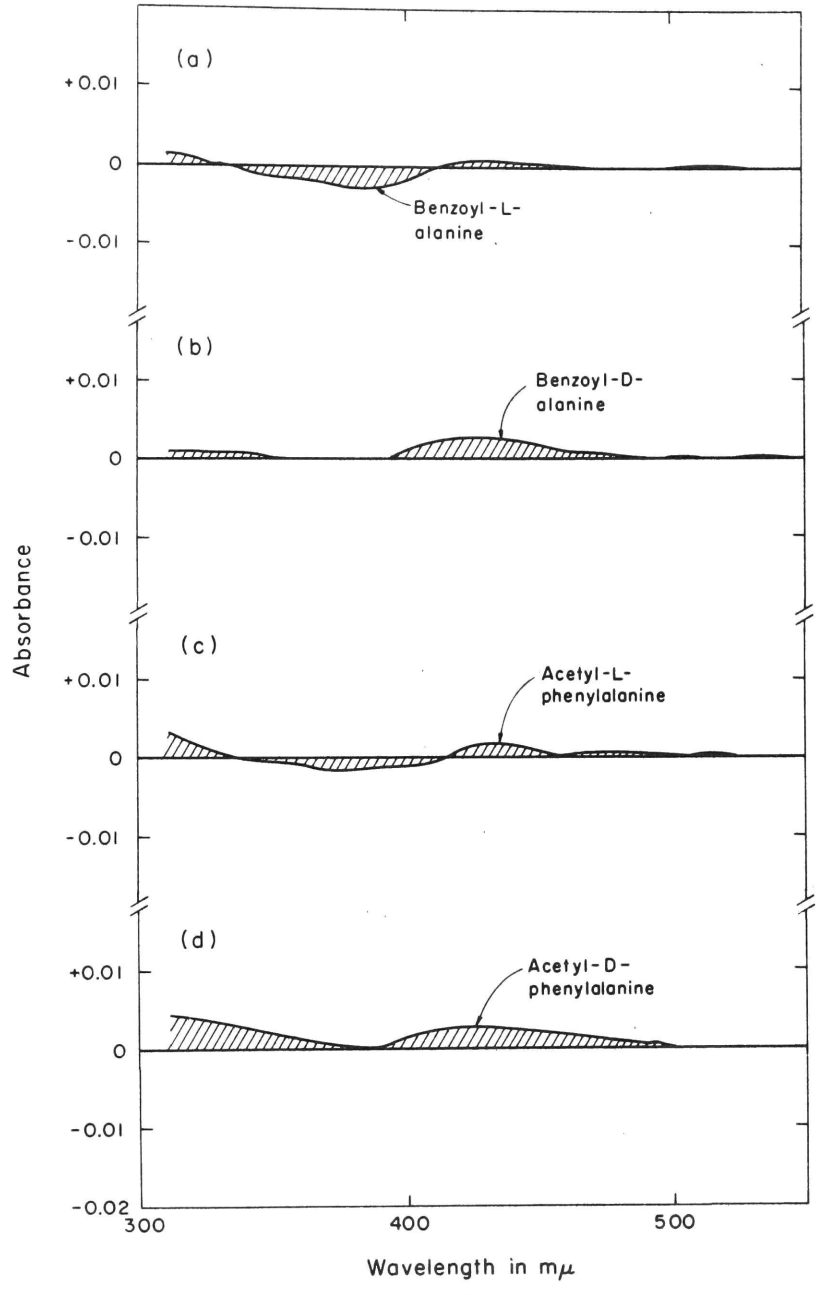

Figure 15 Substrate-generated difference spectra at low concentrations of substrates

Difference spectra of α-chymotrypsin labeled with a reporter group, CT-(R_1-Met_3). Conditions[6]: 7.4×10^{-5} M enzyme, 1.5×10^{-3} M substrate, 0.05 M phosphate buffer, pH 6.49 ± 0.01.

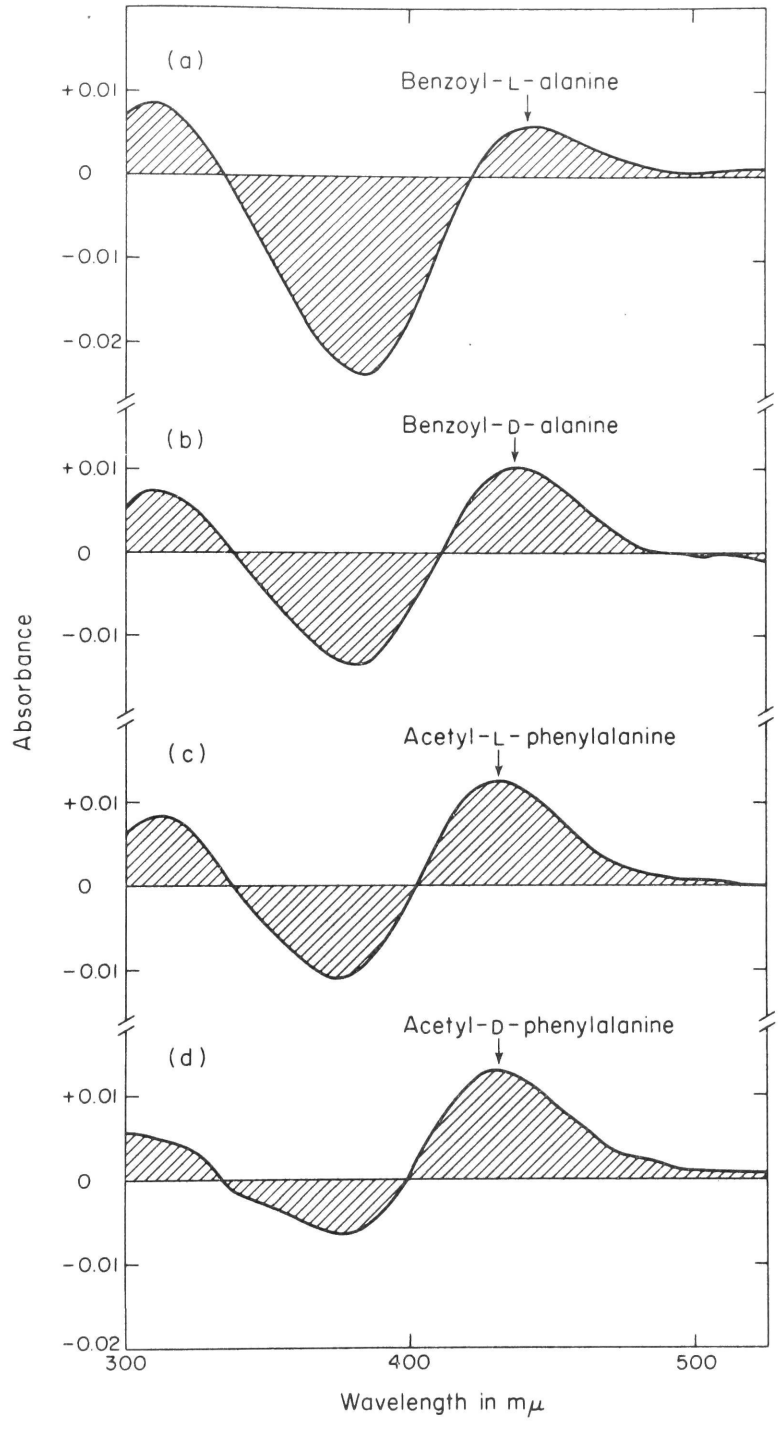

Figure 16 Substrate-generated difference spectra at high concentrations of substrates

Difference spectra of α -chymotrypsin labeled with a reporter group, CT-(R_1-Met_3). Conditions: 3.4×10^{-5} M enzyme, 5×10^{-2} M substrates, 0.05 M phosphate buffer, pH 6.51 ± 0.01, path length 0.88 cm.

benzoyl-L-alanine represents a large decrease in intensity (10%) and a shift of about 4 mμ to a longer wave length maximum. The addition of benzoyl-D-alanine causes a similar shift in the wave length maximum, but the decrease in intensity for the same concentration of substrate is only 4.5%. The smaller effect of the D analogue is not necessarily attributable to binding constants since benzoyl-D-alanine methyl ester has a greater binding affinity to α-chymotrypsin than benzoyl-L-alanine methyl ester. The apparent dissociation constants of these two substrates are 3.3 and 9.7 mM, respectively (Hein and Niemann, 1962a)[7]. Even though the binding constants are probably not the same for the binding of the acid form of the substrates to the reporter-modified enzyme, there is no evidence that the relative rates are different.

Acetyl-L-phenylalanine causes a shift of 4 mμ and a decrease of only about 2% of the enzyme-bonded reporter group spectrum. Acetyl-D-phenylalanine causes a negligible decrease in the intensity of the spectrum. There is also a shift of about 3 mμ to a longer wave length and probably a slight broadening of the spectrum. The larger decrease in intensity by the L isomer is also probably not explained by a lesser binding of the D isomer, since the amide of the D isomer has a more favorable binding constant to α-chymotrypsin (K_m=31 and 12 mM for the L and D amides, respectively; Foster and Niemann, 1955a, b). Of these four substrates, benzoyl-L-alanine causes the largest spectral change.

In summary, analyses of the perturbations induced by substrates in terms of shifts in the wave length maxima of the spectrum and changes in the intensity of the absorption[8] show: (1) that shifts of 1 to 4 mμ to longer wave lengths[9] occur on the addition of substrates and (2) that both positive

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8 General broadening was not considered because the effects are small and not readily analyzed unless very detailed changes in intensity and the shape of the peak absorption of the ionized enzyme-bonded reporter group are first made.

9 A shift of + 12 mμ was observed with 33.3×10^{-3} M phenylpropionic acid and 6.5×10^{-5} M CT-(R_1-Met_3) but it is not certain that this perturbation was due to binding of the substrate at the active site of the enzyme. Drs. H. Strumeyer and D. E. Koshland, Jr. (unpublished) have indications that phenylpropionic acid acts as a detergent on α-chymotrypsin at high concentrations.

and negative changes in intensity up to 10% of the reporter-modified chymotrypsin spectrum occur. The changes are not the same for D and L isomers of the amino acids. In general, the L isomer was found to cause a larger decrease in the intensity of the spectrum. The greater decreases by the L analogues were not necessarily correlated with an increased binding of the L analogues, since the D analogues of many of these substrates bind better to native chymotrypsin. The actual mode of binding of the substrate may, therefore, be important in determining the spectral perturbation.

Shifts in the position of the wave length maximum of R_1 To proceed with the analyses of the observed difference spectra the spectral properties of the reporter group were studied in different environments. The position of the wave length maximum of the absorption peak of ionized R_1H in an aqueous solution, in a 33% solution of dioxane, and of ionized R_1 after it is bonded to α -chymotrypsin were studied over the pH range where this peak is observed.

The absorption maximum for R_1H in 0.05 M phosphate occurs at 410 mμ between pH 7 and 11. The addition of p-dioxane, a compound with a low dielectric constant ($\mathcal{E}$ = 2.21 at 25°) and a refractive index (n) of 1.423, shifts the absorption maximum to about 420 mμ. In changing the solvent from water ($\mathcal{E}$ = 78.5 at 25°, n = 1.333) to one that is more nonpolar in nature and has a higher refractive index, there is a shift to a longer wave length. In changing the aqueous environment of R_1 to that of the enzyme, the shift is about 12 mμ to shorter wave lengths below pH 7 indicating that there is a polar or charged group in the environment of the enzyme-bonded reporter group or an environment with a lower refractive index. The wave length maximum shifts to a longer wave length above pH 7. At pH 11 it is 415 mμ. This change in the wave length maximum with pH indicates that the absorption of the ionized phenolic group is influenced by groups that are charged below pH 7. These groups may be neighboring groups or may influence the conformation of the protein. The environment of the reporter group on the enzyme at high pH appears to be more nonpolar in nature than water.

The addition of substrate to reporter-modified enzyme shifts the maximum of the ionized reporter absorption to longer wave lengths. The maximum occurs between that of the enzyme-bonded reporter group and that of R_1H in aqueous solution for benzoyl-L-phenylalanine from pH 5.7 to 7.5. From evidence that is presented later it appears that the major effect of the substrate is to modify the influence of neighboring ionizable groups on the protein.

Spectrophotometric titrations of R_1H and reporter-labeled α-chymotrypsin Observed changes in the degree of ionization of R_1 in various environments suggested an explanation for some of the substrate-generated spectral perturbations. Spectrophotometric titrations of R_1H in 0.05 M phosphate ions at 410 and 310 mμ shows an increase in the absorption at 410 mμ with increasing pH while that of the 310 mμ peak decreases (Figure 17). The sum of the fractions ionized is always 1.0 ± 0.03. From these data, the absorption of the 410 mμ peak can be attributed to the ionized species and the peak at 315 mμ to the unionized species. The pK of the phenolic group is 6.1, as determined by the midpoint of the titration curve. Data from other wave lenghts on the ionized absorption peak do not differ significantly from that at 410 mμ.

In a 33% (v/v) solution of dioxane in water the pK is 6.5 (Figure 18). The titrations in aqueous solution and in this dioxane solution are identical to the titration of a monovalent weak acid with a strong base.

The titration of enzyme-bonded R_1 is also given in Figure 18. The experimental points are based on the absorption at the maximum. The shape of the curve is changed and the apparent pK is shifted to a more acid value than pH 6.1. The extinction coefficients for the fully ionized form of R_1 at the wave length maximum in 33% dioxane, aqueous solution and on the protein are 1.6×10^4, 1.4×10^4, and 1 to 1.3×10^4. That for the unionized form of R_1H in aqueous solution is 0.4×10^4.

In order to analyze the shift in the absorption maximum and the apparent shift in the pK of the reporter group in the enzyme environment, the absorptions at wave lengths between 330 and 450 mμ were plotted versus pH. These absorptions were normalized at each wave length to their maximal absorptions. From the shapes of the titration curves there seemed to be three dissociating groups with pK values around 5, 8, and 10 that influenced the

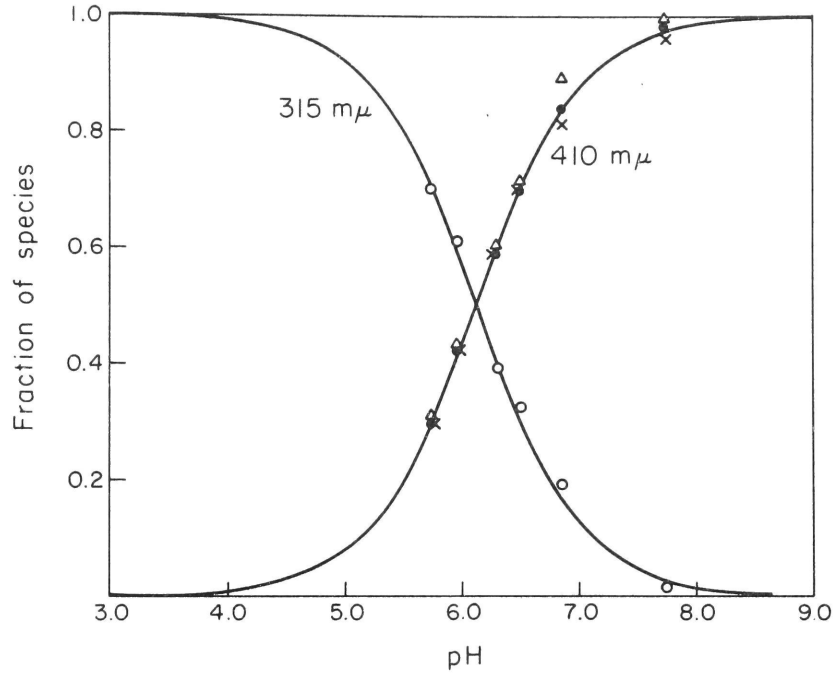

Figure 17 Relative concentrations of unionized and ionized phenolic form of the reporter group

The disappearance of the 315 mμ absorption maximum is shown with the appearance of the 410 mμ absorption peak as the reporter group is ionized. The experimental points were determined at 315 mμ (o), 410 mμ (●), 370 mμ (Δ), and 450 mμ (×). Conditions: 1.3×10^{-5} M R_1H, 0.05 M in phosphate ions.

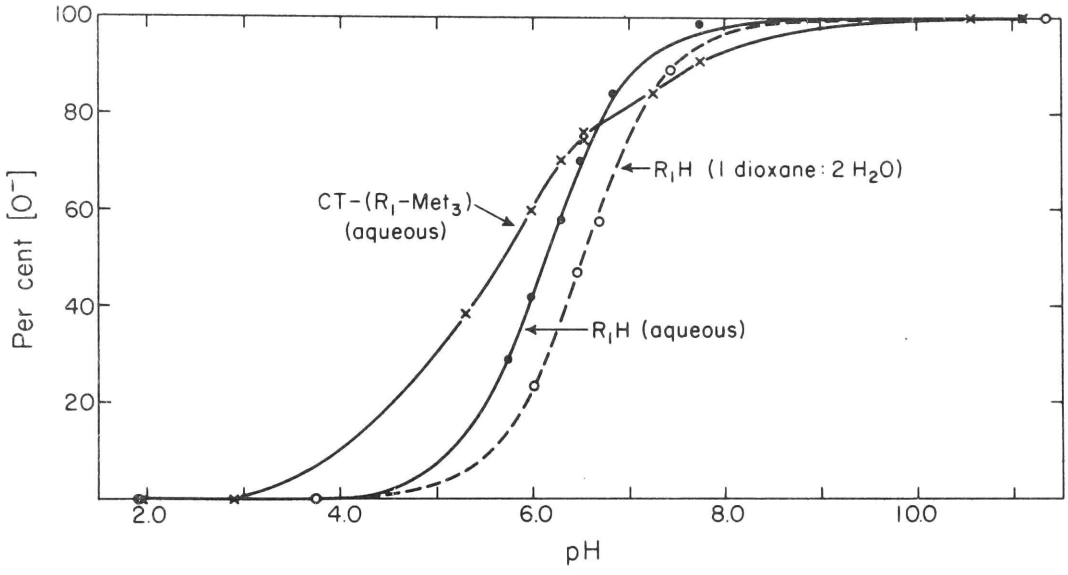

Figure 18 Influence of environment on the ionization of the phenolic hydroxyl of the reporter group

The experimental points are from the absorption peak maximum near 400 mμ. The lines for R_1H in free solution are calculated for the ionization of a monovalent weak acid with a pK of 6.12 for the aqueous solution and a pK of 6.52 for the dioxane water mixture. All aqueous solutions were 0.05 M in phosphate ions.

shape of the titration curves. One pK is that for the dissociation of the phenolic hydroxyl group of the reporter group. The other two must be for groups on the protein that are near the reporter group or influence the environment of the reporter group by changing the conformation of the protein. In several attempts to fit the data, the pK values were estimated to be 5.3, 7.8, and 10.0 $\pm$ 0.2. Since the estimated pK values are more than 2 pH units apart it can be assumed that none of the ionizable species are simultaneously titrated. The reporter group, therefore, must have four different absorption spectra depending on the ionization state of each dissociating group. The four stages of ionization of the enzyme-reporter group complex are represented as A, B, C, and D:

$$A \xrightleftharpoons{pK\ 5.3} B \xrightleftharpoons{pK\ 7.8} C \xrightleftharpoons{pK\ 10.0} D$$

Extinction coefficients for the four forms were calculated in arbitrary units at each wave length from the titration data and from the following equilibrium constants:

$$\frac{(B)\ (H^+)}{(A)} = 10^{-5.3} \quad ; \quad \frac{(C)\ (H^+)}{(B)} = 10^{-7.8} \quad ; \quad \frac{(D)\ (H^+)}{(C)} = 10^{-10.0}$$

Plots of the relative magnitudes of the extinction coefficients versus wave length which are shown in Figure 19 show the estimated absorption curves of each form. The wave length maximum of the A form which undoubtedly is the unionized form of R_1 probably occurs around 320 mμ. Absorption of the protein and of the 285 mμ peak of R_1 contribute to the spectrum at 310 and 300 mμ. The relative extinction coefficient at the peak maximum of the A form is about 0.08 unit. No absolute extinction coefficients are determined since the absolute fraction of R_1 per enzyme molecule is not known. Forms B, C, and D have the following estimated wave length maxima and relative extinction coefficients: B, 395 mμ, 0.14; C, 405 mμ, 0.205; D, 415 mμ, 0.18.

The cyclic product, R_1benzoxamine, produced by the base catalyzed cleavage of R_1 from the methionine of chymotrypsin contributes only a small fraction to the total absorption. The total time that the modified protein was at room temperature above pH 3 was less than 1.5 hr. Since the half life for the cleavage is 15 hr, not more than 5% of the final absorbing forms could be R_1benzoxamine. This product has little absorption at 400 mμ and an extinction coefficient of about 0.4×10^4 from 320 to 380 mμ. The extinction coefficient of R_1H in a basic aqueous solution at 410 mμ is 1.4×10^4.

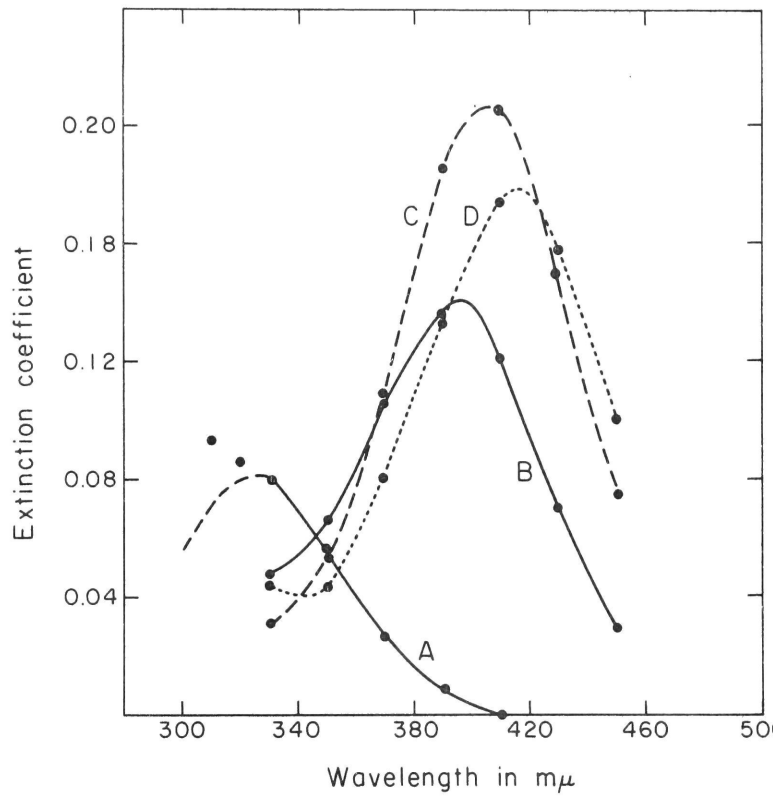

Figure 19 Calculated absorption spectra of the reporter group, R_1, under the influence of three dissociating groups.

These points give the best fit to the data in Figure 20 assuming three dissociating species with pK values of 5.3, 7.8, and 10.0. The extinction coefficients are in arbitrary units.

From the extinction coefficients in Figure 19 and from the equilibrium constants given previously, titration curves at the various wave lengths were calculated. They are the solid lines drawn in the Figure 20. The experimental points also shown in this figure fall very close to the calculated lines. There are no experimental points from pH 8 to 10. In normalizing the experimental absorption values the maximal calculated values were set equal to 1.0. Attempts to fit the data in a similar manner assuming only two dissociating groups were unsuccessful. Also plots for the titration of R_1H in aqueous solution gave a typical curve for the dissociation of a monovalent weak acid.

The presence of substrate changes the shape of the titration curves. When 0.01 M benzoyl-L-phenylalanine is present (Figure 21) the titration curves are steeper around pH 6.0. From pH 8 to 10 there are no experimental points. Since the enzyme is not saturated with substrate the change in the shape of the titration curve is not complete. The shape of the titration curves are compared in Figure 22 with the calculated titration curves for the absence of substrate. The increased steepness of the titration curves around pH 6 indicates that the pK or one or more of the dissociating groups is changed, or that one of these groups is no longer influencing the absorption spectrum of R_1. Another explanation is that a new group with a pK around 6 is influencing the spectrum. In the discussion section it is proposed that the carboxylate anion of the substrate interacts at the active site with one of the dissociating groups that influence the absorption of R_1 in the absence of substrate.

The substrate induced difference spectra could result from changes in the pK values of the dissociating groups and from changes in the influence of each group on the absorption spectrum as well as direct interaction of the substrate with the reporter group. All these effects could change both the intensity and the wave length maximum.

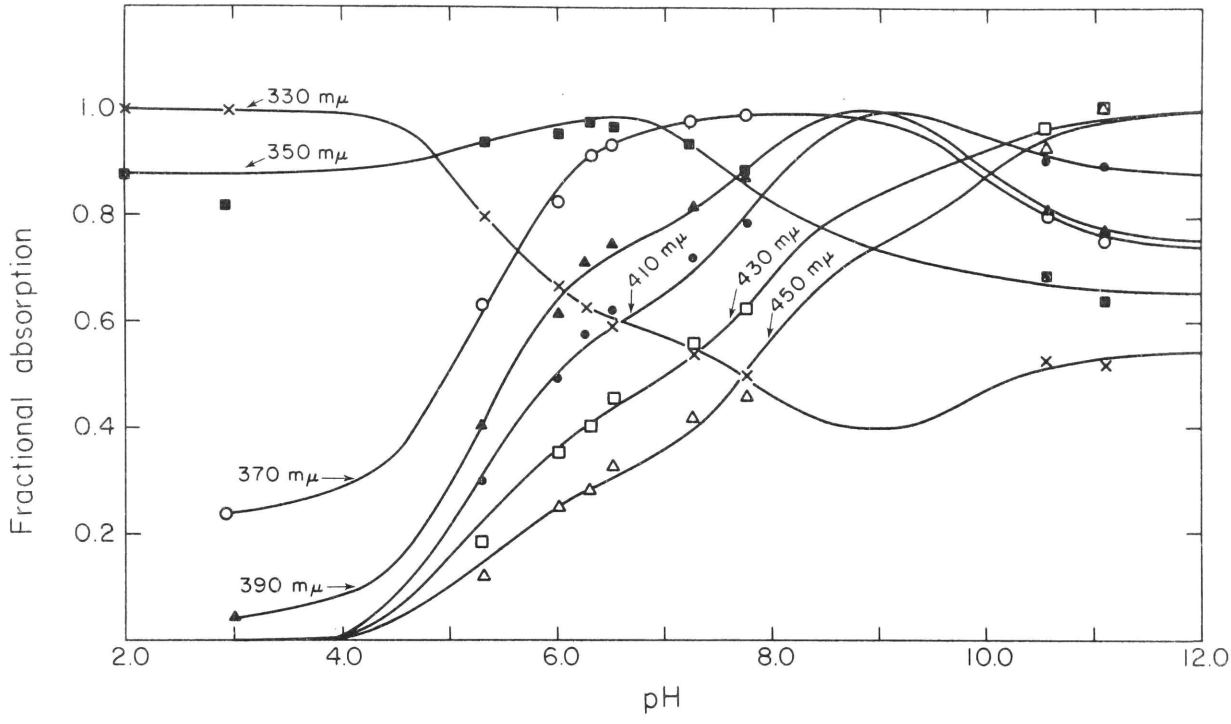

Figure 20 Calculated titration curves and experimentally determined points for the spectrophotometric titration of the reporter group, R_1, bonded to chymotrypsin

The solid lines were calculated from the extinction coefficients in Figure 19 and the equilibrium constants on page 34 assuming that there are three dissociating groups with pK values of 5.3, 7.8, and 10.0. The experimental points are shown for 330 mμ (x), 350 mμ (■), 370 mμ (o), 390 mμ (▲), 410 mμ (●), 430 mμ (□), and 450 mμ (△). Conditions: 2.3×10^{-5} M CT-(R_1-Met_3), 0.05 M phosphate ions.

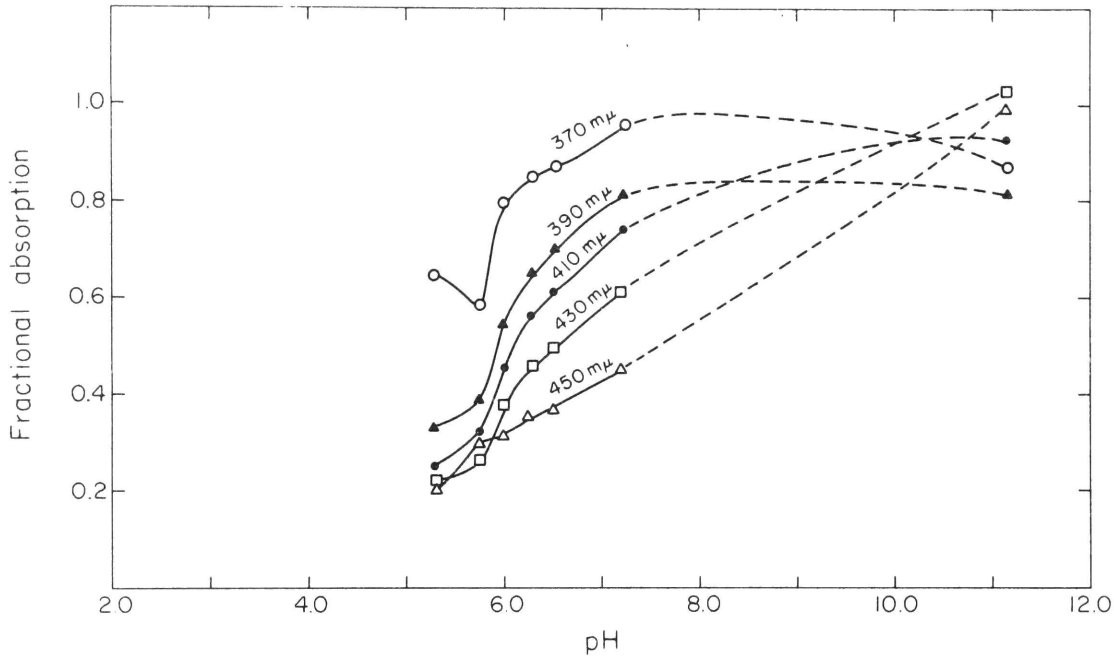

Figure 21 Titration of chymotrypsin-bonded reporter group in the presence of substrate

The experimental points are shown for 370 mμ (o), 390 mμ (▲), 410 mμ (●), 430 mμ (□), and 450 mμ (Δ). The points were normalized to the maximal absorptions in the absence of substrate. Conditions: 2.3×10^{-5} M CT-(R_1-Met_3), 0.01 M benzoyl-L-phenylalanine, 0.05 M phosphate ions.

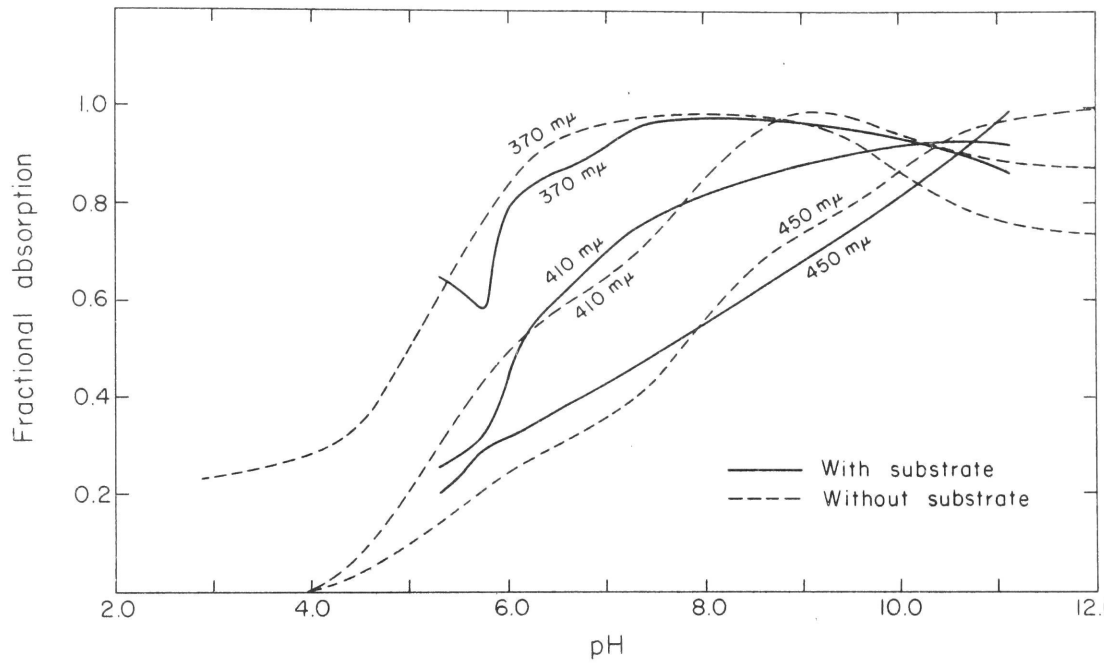

Figure 22 Titration of chymotrypsin bonded reporter group in the presence and absence of substrate

The dotted lines are the calculated titration curves for CT-(R_1-Met_3) in the absence of substrates. The solid lines are from the experimentally determined points in the presence of 0.01 M benzoyl-L-phenylalanine.

Section IV

Discussion

DISCUSSION

The purpose of this work was to place a group which has an environmentally sensitive absorption spectrum at a position near the active site of an enzyme without destroying the activity of the enzyme and to observe changes in the spectrum of this group when substrates bind to the active site of the enzyme. Since it had been established that the methionine three residues from the active serine of chymotrypsin (Met_3) could be modified with only minor changes in the activity of the enzyme (Koshland et al., 1962) a reagent, 2-bromoacetamido-4-nitrophenol, was designed which had both a group with environmentally sensitive spectral properties (a reporter group) and a reactive group (a positioning group) which would direct it to and covalently bind it to this methionine residue. A nitrophenol derivative was chosen because it absorbs in the visible region outside the protein absorption bands, and because its absorption spectrum is sensitive to environmental changes. The bromoacetamide substituent was chosen because it is an analogue of iodoacetic acid which has been shown to react only with the Met_3 in aqueous, pH 3 solutions.

The reporter-modified enzyme registered 100% activity as determined by the "all-or-none" assay with radioactive phenylmethyl sulfonyl fluoride, but showed decreased activity by the ATEE assay. Therefore, each molecule of reporter-labeled enzyme is active but operates at a somewhat reduced rate compared to the native enzyme.

Substrates were demonstrated to have a marked influence on the spectrum of the chymotrypsin-bonded reporter group as is evidenced by the substrate-generated difference spectra (Figures 12 and 15). These spectral perturbations are due to specific changes in the environment of the reporter group. It is the property of the enzyme of specifically binding a substrate molecule to its active site which leads to the change in the environment of the bonded reporter group as measured by the difference spectra. The following observations support these conclusions.

(1) The observed spectral perturbations were not due to changes in pH. The pH was controlled to less than 0.01 pH unit and spectral changes due to this variation in pH were far too small to account for the large changes

in the difference spectra that occurred.

(2) Acetic acid and the substrates have a similar effect on the ionic strength of the medium; however, acetic acid which does not bind to the active site of the enzyme has a negligible effect on the spectrum of the enzyme compared to that of the substrates. It is not, therefore, the change in the ionic strength of the medium that leads to substrate-generated spectra.

(3) Adding substrates to solutions of R_1H or solutions of R_1H and chymotrypsin produces negligible spectral changes compared to the difference spectra measured when substrates are added to solutions of reporter-labeled chymotrypsin. Therefore, at concentrations of the reporter group used, interactions between the reporter group and the substrates or the enzyme-substrate complexes are not large enough to produce a perturbation of the reporter group spectrum unless the reporter group is covalently bonded to the enzyme. Changes in the general medium when substrates bind to the enzyme are also not large enough.

(4) At substrate concentrations less than required for enzyme saturation the spectral perturbation increased with increasing substrate concentration. Increasing the number of enzyme-substrate complexes, therefore, increases the number or reporter molecules perturbed.

(5) The difference spectra produced by the D and L isomers of a substrate have different characteristics. Therefore, the specificity exhibited by the enzyme in binding substrates is important in determining the character of the spectral perturbation.

Titration curves for the reporter group bonded to chymotrypsin can be closely reproduced by assuming that there are three dissociating groups in the enzyme-reporter group complex with pK values of 5.3, 7.8 and 10.0 which influence the absorption spectrum of R_1. Absorption spectra for the 4 forms of the reporter group that occur at each of the ionization states of the reporter environment were calculated to best fit the experimental absorption points using the equilibrium constants for the ionizing species. Form A has an absorption maximum about 320 mμ and a pK of dissociation of 5.3. It undoubtedly is the unionized form of the reporter group. The pK of the reporter group is, therefore, shifted 0.8 pH units to a more acid value on bonding to the enzyme. A neighboring polar or positively charged

group could shift the pK in this manner by stabilizing the ionized form. For instance it has been observed by Benesch and Benesch (1955) that the microscopic pK of the SH groups of cysteine is moved about 1.5 pH units to a more acidic value in the presence of the positively charged amino group. As expected, the microscopic pK of the amino group is also moved 1.5 pH units to a more basic value in the presence of the negatively charged sulfhydryl group.

The absorption of the ionized form of the reporter group phenol is influenced by a group on the enzyme that dissociates at pH 7.8 and one that dissociates at pH 10.0. The histidine residue that has been identified as part of the active site has in unmodified chymotrypsin a pK of 7 and is positively charged below pH 7. The imidazole cation of the histidine could be the group that lowers the pK of the reporter group to 5.3. Both groups are at or near the active site. The negatively charged phenolic group of the reporter group would simultaneously shift the pK of the histidine to a more basic value. The shift would be about the same magnitude as that for the reporter group. Since the pK of the histidine in the presence of the ionized phenol would be 7.8 or identical to that of one of the observed ionizing groups we have identified it as this group. The ionized form of the reporter group absorbs as the B form in the presence of the positively charged form of the imidazole group of the histidine and as the C form in the presence of the neutral form of the imidazole group. The positively charged imidazole shifts the spectrum for the B form of the reporter group to a shorter wave length than that of the C form. As expected this shift is in the opposite direction from that observed when dioxane is added to a solution of R_1H.

The fourth form, D, is present after a group with a pK of 10 has ionized. α-Amino terminal residues and the ϵ-amino group of lysine residues have pK values around 10. Tyrosine residues also have pK values around 10.9. The inactivation of chymotrypsin is influenced by a group with a pK around 10 (Kunitz and Northrop, 1935) which is believed to contribute to the stability of the protein. Labouesse, Oppenheimer, and Hess (1964) communicated that the dissociation of the α-amino group of the isoleucine of the B chain influences both the specific rotation (α) of chymotrypsin and the rate of catalysis. They proposed that this group maintains the protein in a catalytically active conformation since acetylation of this group results in

an inactive protein.

It is likely that the reporter group which was designed to note changes at the active site of chymotrypsin are influenced by the ionization state of amino acid residues at the active site or by amino acids that influence the conformational stability of the active site. The assumptions that there are two groups on the protein with pK values around 7.8 and 10.0 that influence the absorption of the enzyme-bonded reporter group are supported both by the fit of the data to the calculated curves and by the evidence that two groups with similar pK values influence the activity of the enzyme. The histidine residue at the active site has been suggested as the group that dissociates at pH 7.8.

The addition of the substrate benzoyl-L-phenylalanine to solutions of reporter-modified chymotrypsin appears to shift the pK of the phenolic group of the reporter group to a higher pH. The effective charge of the histidine cation must, therefore, be less when the acid form of the substrate is bound to the active site. The influence of the substrate on the charged histidine group can be explained by an electrostatic interaction of the substreates' negatively charged carboxyl group with the histidine which neutralizes the positive charge, or by a conformational change at the active site on the binding of the substrates which increases the distance between the phenolic group and the positively charged group. Since Schoellmann and Shaw showed that the histidine is in the correct position to react with the alcoholic oxygen of the ester bond of the substrate when the substrate is bound to the active site, the most reasonable explanation of the effect of the substrate on the positive charge is that the substrate nulifies the effect of the positive charge of the histidine.

Careful analysis of the difference spectra in terms of changes in the absorption maximum and the intensity of absorption when substrates are added indicate that different substrates do not produce the same magnitude or pattern of changes. The variation in these perturbations does not appear to be related simply to binding constants. The contribution of the different substituents of the substrates to the immediate environment of the reporter group is not necessarily the same. Also, the influence of the substrates on the pK of the histidine or its effective charge need not be identical. The evidence that the D and L configurations of some substrates

lead to different spectral perturbations indicates that they do not bind in the same manner.

A consistent interpretation of the substrate-induced perturbations in terms of the three-dimensional arrangement of the substrates at the active site has not yet been accomplished. However, the variations in the perturbations caused by the different substrates, the influence of the substrate's configuration on the perturbation, and the sensitivity of the reporter group to environmental changes and to the addition of substrates, together with the evidence that the data is amenable to a systematic interpretation, make us hopeful that application of this technique to the orientation of substrates at the active site will be possible.

From these studies with R_1-modified α-chymotrypsin some of the limitations and possible future applications of the reporter group method are indicated. Two of the limitations of the reporter group reagent, 2-bromoacetamido-4-nitrophenol, are that it is not very soluble in aqueous solutions and that both the reagent and its methionine derivative cyclize in neutral or basic solutions. In these studies we were able to minimize these problems. The solubility problem was overcome by using a methanolic solution and long reaction times at room temperature. This was possible since the loss of activity of α-chymotrypsin in 20% methanol at pH 3 is small even when at room temperature for periods of weeks. In using methanolic solutions the possibility of reactions with the inside methionine (Met_{15}) is introduced if the configuration of the protein changes in methanolic solutions. If necessary chromatographic purification of a homogeneous protein, as has been accomplished for the monosubstituted derivatives of ribonuclease (Crestfield et al., 1963a, b), seems possible. The position of the reporter group will have to be identified before the final interpretation of the difference spectra are made. Nevertheless, the exact position does not alter the significance of our positive result that substrates induce spectral changes of the enzyme-bonded reporter group. Cyclization of the reporter group reagent did not affect these studies since they were carried out at pH 3 where little or no cyclization occurs. The cyclization of the 2-acetamido-4-nitrophenol on methionine at neutral or alkaline pH values was sufficiently slow and resulted in a colorless product so that interference with the spectral measurements could be avoided.

A limitation in the interpretation of the substrate-induced spectral shifts exist since there is no direct evidence that the reporter group is at the active site. The difference spectra could be caused by contact of the substrates with the reporter group or by changes in the conformation of the enzyme near the reporter group that are induced by the binding of substrates to the active site. Eventual knowledge of the three-dimensional structure of the protein from X-ray data may resolve this problem. For example, the reporter group may be found to be so far from the active site that direct interaction with the substrate is impossible. The interpretation of substrate-induced spectral changes are also limited by the absence of a sound theoretical basis for the effect of solvents on absorption spectra.

The application of this technique to the study of conformational changes has been suggested. Positioning groups with different specificities might be used to map out the areas where conformational changes occur. The areas of protein-subunits where associations occur might also be detected.

These studies indicate that the reporter group method has potential for detecting environmental changes at specific positions in protein molecules and that meaningful empirical correlations are possible. The environmental changes may be caused by direct substrate interactions or by indirect conformational changes. A description of the three-dimensional orientation of substrates at the active site may eventually be possible with this method.

Section V

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