The Formation of Anhydrochymotrypsin by Removing the Elements of Water from the Serine at the Active Site[1]

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Abstract: The serine residue at the active site of chymotrypsin has been converted to a dehydroalanine residue by (a) tosylating the active serine and only the active serine and (b) catalyzing an elimination reaction by treatment of the tosyl enzyme with alkali. The new enzyme, "anhydrochymotrypsin," was inert. Control experiments established that the loss of the hydroxyl group of serine, not the conditions of the treatment, were responsible for the loss in activity. The presence of the dehydroalanine residue was established by identification of addition products, chemical analysis, and spectrophotometry. The physical properties of the anhydrochymotrypsin were essentially the same as the native enzyme including the ability to bind substrates and inhibitors. These experiments remove the "steric access" ambiguity in relation to the role of serine and establish that it plays an essential role in the catalytic function as compared to the specificity function of the enzyme.

As our knowledge of physical organic mechanisms increases and the three-dimensional structures of proteins become known precisely, the importance of establishing the detailed role of an amino acid residue in enzyme action increases steadily. Although a SH was implicated at the active site of succinic dehydrogenase in 1938,[5] the first residue to be identified with the catalytic activity of a protein was undoubtedly the serine of chymotrypsin.[6] That the precise role of serine is still a source of controversy[7-12] is an indication of the complexities and challenges in defining the roles of amino acid residues. The importance of resolving the role of serine is emphasized by its implication in the action of many other enzymes[13-16] and the finding that other residues, *e.g.*, lysine and cysteine, appear to have roles analogous to serine in the action of other enzymes.[17-20]

The controversy surrounding serine can probably be illustrated with the aid of Figure 1 which shows the major components—serine, histidine, water, substrate—of the reaction catalyzed by chymotrypsin. The model represented in Figure 1 schematically summarizes the universally accepted facts that (a) one histidine residue is essential for catalysis and is in the region of the active site, (b) the ultimate acceptor of the acyl group of the substrate is water, and (c) a serine residue must be at or near the active site. The controversy revolves around the question of whether serine is an essential nucleophile during the normal action of chymotrypsin or whether its apparent involvement is an artifact induced by abnormal conditions.

Certain lines of evidence point strongly to the universal formation of an acyl enzyme intermediate, dividing the catalysis sequence into at least three steps: (1) an initial adsorption of substrate (eq 1), (2) formation of an acyl enzyme (eq 2), and (3) the deacylation

$$E + AcX \longrightarrow E\cdot AcX \quad (1)$$

$$E\cdot AcX \longrightarrow EAc + X \quad (2)$$

$$EAc + H_2O \longrightarrow E + AcOH \quad (3)$$

of this acyl intermediate through reaction with water (eq 3). Among the arguments favoring this sequence are the observation of acyl or phosphoryl intermediates,[6,10,11] the identical hydrolysis rate of various ester substrates,[21] and the pH dependence of the reaction.[22]

An alternative proposal places serine at the active site but postulates that the hydroxyl group of serine competes with water in the attack on the carbonyl group of the substrate. When it does so, an acyl intermediate is formed. The deacylation of this intermediate would then occur in precisely the same way as the hydrolysis of the initial substrate, *i.e.*, by activation of a water molecule (*cf.* eq 4). This alternative suggests that serine is part of an alternate pathway which could vary from 0 to 100% depending on the conditions but

(1) Preliminary accounts of this work have been published.[2,3] Abbreviations: anhydrochymotrypsin, chymotrypsin in which the active serine has been converted to dehydroalanine; base-treated chymotrypsin, native chymotrypsin which has been treated with 0.1 *N* NaOH for several hours at 0°, *i.e.*, the conditions required to produce anhydrochymotrypsin; tosylchymotrypsin, chymotrypsin containing one tosyl group per molecule of enzyme; ATEE, acetyltryosine ethyl ester; DPNH, reduced form of diphosphopyridine nucleotide: DIP-chymotrypsin, diisopropylphosphorylchymotrypsin; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

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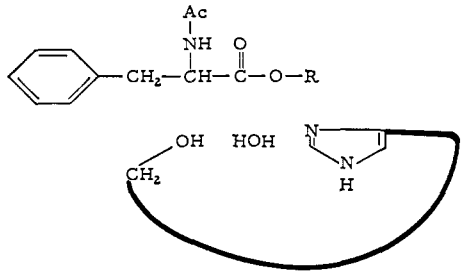

Figure 1. Major components implicated in the active site during enzyme action of chymotrypsin.

is not an obligatory intermediate in the hydrolysis of all substrates. Arguments for this position are (a) that the acyl intermediates can be detected only under abnormal conditions, *e.g.*, abnormal substrates such as nitrophenylacetate or abnormal pH such as pH 3, (b) that quenching experiments fail to establish the calculated stoichiometry of acyl intermediates,[8,9] and (c) that the kinetics of amide substrates do not require any such intermediates.[7,8]

$$\begin{array}{c} EAc + X \\ \uparrow\downarrow \quad \searrow^{H_2O} \\ E + AcX \longrightarrow E\cdot AcX \xrightarrow{H_2O} E + AcOH + HX \end{array} \qquad (4)$$

Since all the modifications of the serine residue used so far increase its size appreciably, and there is strong support for the catalytic role for histidine,[10,12,23,24] it is argued that modification of serine produces inactivity, not because the serine itself plays a vital role but because the increase in size prevents access of the substrate to the true catalytic residue, *e.g.*, histidine. It therefore seemed desirable to produce a modification of serine which involved a major change in its chemical properties but which did not add significantly to its steric size.

Conversion of the serine residue to a smaller residue, dehydroalanine, was achieved by the sequence of steps shown in eq 5. Conversion of serine to dehydro-

$$\begin{array}{l} CH_2OH \\ | \\ -CH- \end{array} \longrightarrow \begin{array}{l} CH_2O-SO_2-\langle\rangle-CH_3 \\ | \\ -CH- \end{array} \longrightarrow \begin{array}{l} CH_2 \\ \| \\ -C- \end{array} \qquad (5)$$

alanine by an elimination reaction appeared possible following the work of Photaki on model serine peptides.[25] Tosyl chloride was initially used to convert the serine to a tosyl derivative.[2] However, Fahrney and Gold[26] demonstrated by using phenylmethanesulfonyl fluoride that the fluoride derivative was more selective. Thus in later work tosyl fluoride was used to label the active serine without modification of any other residue. The elimination of the tosyl group was effected by a base-catalyzed reaction to produce "anhydrochymotrypsin," so named because the elements of water have been removed from the serine at the active site.

If the serine hydroxyl performs an essential role in enzyme activity, the anhydrochymotrypsin would be expected to be inert. If the serine acts merely as a competitor with water for the acyl group of the substrate, then the conversion should not affect the rate of the reaction. The experiments and conclusions derived from the preparations and properties of anhydrochymotrypsin are reported below.

Experimental Section

Preparation of Tosyl Fluoride from Tosyl Chloride. Tosyl chloride (0.24 g), 0.33 g of sodium fluoride, and 0.4 ml of dimethylformamide were mixed together and stirred for 10 min at room temperature. The mixtures did not become homogeneous but became orange colored. The mixture was then placed in a preheated oil bath at 110–115° for 3.5 hr with stirring. Then 7 ml of water was added and the solution was extracted five times with 4 ml of ether. The ether solution was dried over calcium chloride, the ether was removed, and the solid material was dissolved in 1 ml of 30–60° petroleum ether. On placing this material at about −20° overnight the material crystallized and was collected on a sintered glass filter. The yield of 0.104 g was approximately 35%, mp 41.5–42°.[27] Radioactive tosyl chloride was converted to the fluoride by these procedures.

Preparation of Tosylchymotrypsin. α-Chymotrypsin was obtained from the Worthington Corp. and was treated without further purification. In a typical experiment, 0.4 ml of 3.4×10^{-2} *M* tosyl fluoride in dioxane was added to 1 g of chymotrypsin in 30 ml pH 7.0, 0.05 *M* phosphate buffer at room temperature. After 15 min the enzyme was assayed for activity. At this stage a second aliquot (0.4 ml) was added to the solution and again assay for activity was made. This was repeated until the enzymatic activity was reduced to approximately 0.1–0.5%. Usually five additions, involving approximately a 50% excess of tosyl fluoride, were required to reduce the activity to this level. The resulting solution was then subjected to gel filtration on a 60 × 3.5 cm Sephadex G-25 column and eluted with water. The yield was approximately 900 mg.

Formation of Anhydrochymotrypsin from Tosylchymotrypsin. A typical elimination reaction was performed by treating 400 mg of tosylchymotrypsin at concentrations of either 1 mg/ml or 8 mg/ml with 0.1 *N* sodium hydroxide at 0° for 4 hr. At the end of this period the pH was adjusted to 7.0 with either acetic acid or hydrochloric acid and the solution was subjected to gel filtration on a Sephadex G-25 column.

Equilibrium Dialysis. Equilibrium dialyses were performed according to standard procedures but certain specific precautions had to be taken. To prevent decomposition of proflavine which is light sensitive, dialysis cells were protected from light during equilibration and subsequent manipulations prior to assay. To correct for proflavine bound to the cellophane membrane, actual concentrations of proflavine in each cell component were assayed rather than subtracting the contents of one compartment from the amount initially added.

During the 44-hr interval of equilibration it was observed that some autolysis occurred and that approximately 5% of the chymotrypsin as measured by absorbance had migrated to the nonprotein compartment. Correction for this loss was made before the final calculations.

The binding constant of proflavine was determined by the equilibrium dialysis procedure of Klotz, *et al.*[28] The dissociation constants of substrates and inhibitors which could not be examined directly were established by competitive dialysis procedure[29] and were calculated by the means of eq 6. In this equation the quanti-

$$K_S = \frac{K_{In}[E\cdot In][S]}{[E_T][In] - K_{In}[E\cdot In] - [E\cdot In][In]} \qquad (6)$$

ties $[E_T]$, [In], and [E·In] refer, respectively, to the total enzyme concentration, the free proflavine concentration, and the enzyme-bound proflavine concentration. [S] refers to the concentration of added substrate or inhibitor which is in such excess that its concentration is essentially constant in both compartments. The value

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of the proflavine dissociation constant (K_{In}) from native α-chymotrypsin was previously determined to be 2.5×10^{-5} *M*.[3]

Enzyme Assays. The standard acetyltyrosine ethyl ester assay was performed by a slight modification of the procedure of Schwert and Takenaka.[30] The all-or-none assays[31] were performed to establish what fraction of the molecular species present had some activity. Either the spectrophotometric method using cinnamoylimidazole[32] or the tosyl fluoride-C^{14} assay was used.[33]

Pyruvic Acid Assay. To assay for dehydroalanine the protein solution was treated to convert the dehydroalanine residue to pyruvic acid and the pyruvic acid produced was measured with the aid of lactic dehydrogenase. The conversion of dehydroalanine in the protein to pyruvic acid followed the procedure of Patchornik, *et al.*[34] The protein solution was heated with 3 *N* HCl for 1 hr in a boiling water bath, the pH was then adjusted to 7.5 using concentrated NaOH, and the volume adjusted to exactly 5 ml using 0.1 *M* phosphate buffer, pH 7.5. During the hydrolysis it was occasionally necessary to stir or shake the flask to ensure a homogeneous solution.

A sample was then added to a Beckman cell containing 0.1 *M*, pH 7.5, phosphate buffer. To the sample cell was added 0.1 ml of a 1-mg/ml DPNH solution and the change in absorbance at 340 mμ was recorded. To both sample and reference cells was added 0.1 ml of a 0.2-mg/ml muscle lactic acid dehydrogenase solution and the absorbance at 340 mμ was followed until a maximum value was reached (~2–3 min). In general it was found that 2-ml aliquots were optimal and that a slope of 3.5×10^{-3} mg of pyruvic acid per 0.1 absorbance unit was obtained in the assay.

Kinetics of Tosyl Elimination from Tosylchymotrypsin. Aliquots (1 ml) were removed from the radioactive protein solution and added to 3 ml of 20% trichloroacetic acid at 0°. After standing at 0° for 6–24 hr, the precipitate was collected by centrifugation and washed successively with 3 ml of 5% trichloroacetic acid, ethyl alcohol, 50:50 alcohol–ether, and finally pure ether. The precipitate was then dissolved in a minimum of formic acid, plated on a planchet that had been washed with methanol, and dried under a heat lamp. Radioactivity was measured in a proportional counter.

Results

Elimination of Radioactivity from Tosylchymotrypsin. When tosylchymotrypsin was prepared from tosyl chloride, reaction occurred not only at the active site but also at some other positions in the molecule as indicated by the elimination reaction (*cf.* Figure 2A). The radioactivity present was attached at least at two different sites. From one it could be removed fairly rapidly with a half-life of approximately 1 hr and from the second it was not removed at all under these conditions. When tosyl fluoride was incubated with the enzyme, stoichiometric reaction occurred yielding 1 mole of tosyl group per mole of chymotrypsin, and this radioactivity could be completely removed in a first-order reaction (Figure 2B). If the total radioactivity in the tosyl chloride labeled enzyme is corrected by subtracting out the component which is inert to base, the remaining radioactivity falls on a first-order plot which is strikingly similar to the rate of elimination of radioactivity from the tosyl fluoride labeled enzyme (Figure 2C). It seems clear that tosyl chloride reacts both at the active site of serine and at some other position, possibly a lysine residue, the latter position being inert to base under these conditions. This specificity of the fluoride relative to the chloride is in agreement with results of Fahrney and Gold who first showed the better selectivity of the fluoride using phenylmethanesulfonyl fluoride.[26]

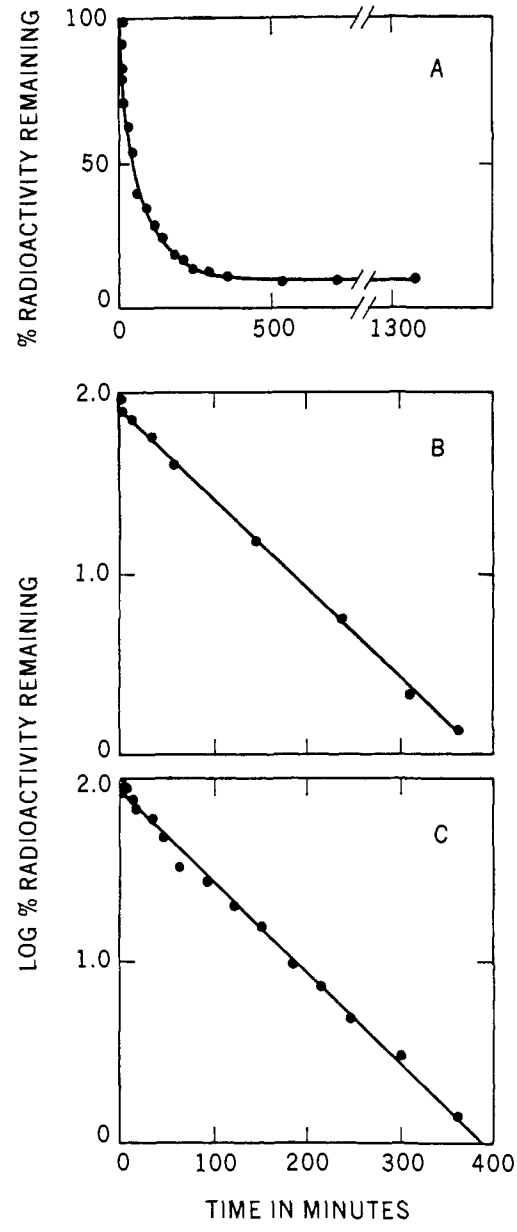

Figure 2. Rate of loss of radioactivity from tosylchymotrypsin: (A) loss of tosyl group from chymotrypsin labeled with C^{14}-tosyl chloride until 1.1 moles of tosyl/mole of enzyme present; (B) loss of tosyl groups from chymotrypsin labeled with C^{14}-tosyl fluoride until 1.0 mole of tosyl/mole chymotrypsin; (C) loss of tosyl groups from tosyl chloride labeled enzyme of Figure 2A if final inert label is subtracted from total present at time *t*.

Close examination of Figures 2B and 2C shows a small initial burst followed by a clean, first-order reaction. Repetition of the experiment many times always gave the same result. Since the chemical assays and stoichiometry strongly suggest that a single serine residue is tosylated, some explanation such as a conformational change on exposure to alkali might be a plausible explanation for the more rapid initial reaction. However, no definitive explanation can be given at the present time. The velocity of the reaction with alkali is comparable to the rates of elimination reactions of model serine peptides.[25,35]

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Table I. Effect of Conditions on Rate of Loss of the Tosyl Group from Tosylchymotrypsin

Concn of OH^-, M	Time	Temp, °C	% C^{14}-tosyl group remaining in protein after treatment
10^{-1}	55 min	0	50
10^{-1}	17 min	22	20
10^{-4}	3.5 hr	22	85
10^{-4}	3.5 hr	35	45
10^{-7}	20 hr	35	100

50%. The positions of the cysteic acid and carboxymethyl cysteine on the amino acid analyzer were already known. The position of the S-phenylcysteine was found to be before tyrosine from the analysis of a synthetic sample. The three reagents were then incubated with anhydrochymotrypsin and the protein was hydrolyzed with HCl in the usual manner. As a control the compounds were also incubated with "base-treated chymotrypsin," *i.e.*, native chymotrypsin which had not been tosylated but which had been given the same base treatment at 0° in 0.1 N OH^- as the tosyl enzyme. The yields as determined on the amino acid analyzer are given in Table II. Assuming an average

Table II. Adducts Obtained on Treatment of Chymotrypsin Preparations with Reagents Known to Add to Double Bonds

Reagent	Time of reaction, hr	pH of reaction	Adduct found	Moles of adduct/mole of enzyme: Base-treated enzyme	Moles of adduct/mole of enzyme: Anhydro-chymotrypsin	Estimated moles of dehydroalanine[a]
Na_2SO_3	20	9.0	Cysteic acid	0.21	0.56	1.0
Thioglycolic acid	24	8.2	Thioglycolic acid	0.55	0.95	1.2
Thiophenol	26	9.4	S-Phenylcysteine	0.20	0.44	.75

[a] Estimated assuming the average yield of 33% from the model experiments and multiplying this yield factor by difference between anhydro and base-treated enzyme yields.

It was of interest to observe the rate of elimination under other conditions, and therefore tosyl-C^{14} enzyme was incubated at various temperatures and times. Some representative values are shown in Table I. From these data it is clear that the use of hydroxide ion concentrations appreciably lower than 0.1 M caused a significant decrease in the rate of elimination of the tosyl group from chymotrypsin. A higher temperature did result in some increased rate of elimination relative to the values at 0°. However, control experiments with native enzyme at 20° indicated appreciable loss of native enzyme activity and therefore these conditions were unacceptable for the present investigation. Experiments of this sort indicated, therefore, that the optimal conditions for preparation of anhydrochymotrypsin from tosyl enzyme involved 0° and hydroxide ion concentrations of the order of 0.1 M.

Since the reaction is undoubtedly a base-catalyzed elimination, the use of other bases to augment the rate of elimination has been considered and the results of preliminary experiments are promising. Moreover, phenylmethanesulfonyl enzyme appears to eliminate more rapidly than the tosyl enzyme. Thus, milder conditions for producing anhydro chymotrypsin may be found by further variations of this type.

It is interesting that the elimination reaction predominated over oxazoline formation in the protein whereas Ginsberg and Wilson[35] found that serine peptides favor oxazoline formation relative to dehydroalanine formation.

Chemical Evidence for the Formation of Dehydroalanine. To obtain more positive proof of the formation of dehydroalanine, adducts were formed using reagents known to add to acetyldehydroalanine. Sodium sulfite, thioglycolic acid, and thiophenol all were added to the double bond of acetyldehydroalanine to produce cysteic acid, carboxymethylcysteine, and S-phenylcysteine, respectively, in yields of from 30 to yield of 33% based on the results with acetyldehydroalanine, a calculated value of approximately 1 mole of dehydroalanine per mole of protein was obtained. Although the quantitation was only approximate, the formation and yields of the new derivatives showed that dehydroalanine was present in the anhydro enzyme and that the major fraction of the tosylated serine residue has been converted to dehydroalanine.

It is to be noted that some cysteic acid, carboxymethylcysteine, and S-phenylcysteine were also formed in the base-treated chymotrypsin, and two sources seem chemically reasonable. Some elimination reaction may occur at the many serine and cysteine residues throughout the protein. In addition there may be direct displacement reactions at the cysteine residues. Although the reaction at each of these residues was far slower than that of the tosylated serine, the total number of groups was greater, *i.e.*, 20 serine and 10 cysteine residues, and hence might be expected to add up to 0.2 to 0.5 residue.

Further support for the presence of a dehydroalanine residue was obtained from the spectral change in the 240-mμ region. Acetyldehydroalanine showed a strong absorption in this region. A difference spectrum of native chymotrypsin *vs.* anhydro enzyme gave a single, sharp peak in the 240-mμ region with an extinction coefficient of 10^4. A rough estimate of 0.5–1.5 moles of dehydroalanine can be obtained from this extinction coefficient but the quantitation can again only be considered approximate since the absorption spectra of model compounds[36] showed a great sensitivity to the substitutents on adjacent atoms.

Pyruvic Acid Assay for Dehydroalanine. When acetyldehydroalanine was analyzed, the yield of pyruvic acid was found to be between 97 and 100%. However, if native chymotrypsin was added to a solution of

(36) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, p 858.

acetyldehydroalanine and the same procedure was followed, the yield of pyruvic acid decreased with increasing concentrations of the protein. The effects of added protein can be seen in Table III. The decrease in yield of pyruvic acid was an approximately linear function of protein concentration at constant pyruvic acid concentration. If the amount of protein was kept constant and the dehydroalanine content was varied, a similar result was obtained. These two experiments indicate either that acetyldehydroalanine or pyruvic acid is reacting with some residue of the protein during hydrolysis. To clarify the nature of the reaction further, a mixture of all the amino acids in chymotrypsin was mixed with acetyldehydroalanine and incubated with the hydrochloric acid solution under the same conditions as used in the hydrolysis experiments. No significant reaction with any of the amino acids could be detected under these conditions. Apparently special conditions present in protein hydrolysis must be responsible for the special reactivity of the protein with the dehydroalanine. Since it is clear that the yield of pyruvic acid was a function of the concentration of the protein present, correction for this side reaction was made using the yields obtained in the experiments of Table III.

Table III. Yield of Pyruvic Acid on Hydrolysis of Acetyldehydroalanine in the Presence of Chymotrypsin[a]

Initial dehydroalanine concn, $M \times 10^5$	Chymotrypsin concn, $M \times 10^5$	Yield of pyruvic acid, %
4.68	None	97.1
2.34	4.9	88.3
3.51	4.9	82.3
4.68	4.9	84.7
7.04	4.9	80.9
9.0	None	97
9.0	0.9	99
9.0	1.7	94
9.0	4.2	91

[a] 100°, 3 *N* HCl, 1 hr.

Dehydroalanine Content of Anhydrochymotrypsin. The pyruvic acid yields from acid hydrolysis of anhydrochymotrypsin and of various control preparations are shown in Table IV. When native chymotrypsin was assayed by the pyruvic acid procedure outlined in the Experimental Section, 0.2 mole of pyruvic acid was produced per mole of enzyme. With "base-treated" chymotrypsin approximately 0.6 mole of pyruvic acid per mole of enzyme was obtained. The anhydro enzyme contained 1.4 moles per mole of enzyme. The 0.2 mole observed in native enzyme is probably produced by acid-catalyzed elimination reactions of serine and cysteine residues during the HCl treatment of the protein. The increase to 0.6 mole when native chymotrypsin is treated with base is probably caused by base-catalyzed elimination reactions of serine and cysteine residues. The number 0.6 is therefore a control value which includes both the nonspecific elimination reactions during the base treatment and the acid-catalyzed eliminations during the assay.

Table IV. Pyruvic Acid Yields on Acid Hydrolysis of Chymotrypsin Preparations

Chymotrypsin preparation	Pyruvic acid, moles/mole of enzyme
Native	0.2
Base treated	0.6
Anhydro	1.4
Acid-treated tosyl	0.2
Tosyl	0.3

It is clear that the anhydro enzyme contains at least 0.8 more residue of dehydroalanine than the base-treated native enzyme and that the source of the increase is the elimination of the tosyl residue from the serine at the active site. In view of the precise stoichiometry with the C^{14}-tosyl fluoride indicating the presence of 1 mole of tosyl group per mole of enzyme, it is quite possible that a stoichiometric conversion to dehydroalanine has occurred at the active site. If, for example, the active site serine in the native enzyme is unusually reactive in base elimination, the figure 0.6 may be too high to use as a control value. Also in the control experiments of Table III acetyldehydroalanine was added externally to a protein solution. Thus, the intimate intrarelationship between the enzyme and the dehydroalanine residue that occurs when anhydrochymotrypsin is treated with acid would not be duplicated. At this point, therefore, it can be said that at least 80 and possibly 100% of the protein has been converted to "anhydrochymotrypsin" by the tosylation and elimination procedures.

Possible Side Reactions during Base Treatment. Because the pyruvic acid analysis was not 100%, a search for possible side reactions was made. One of the most obvious would be the production of lysinolysine which was observed by Bohak.[37] Anhydrochymotrypsin was hydrolyzed in acid and the hydrolysate was placed on the amino acid analyzer[38] to search for the lysinolysine adduct. No significant concentration of this adduct was observed. In 2 hr at a temperature of 40° Bohak observed that chymotrypsinogen gave approximately 2.6 residues of the lysine adduct. There are two sequences involving adjacent lysine–cysteine residues in chymotrypsinogen: one at the positions 168–169 and the other at positions 201–202 in the Hartley sequence.[39] It is conceivable that minor amounts of the lysinolysine adduct are produced under the mild elimination conditions in the experiment reported here, but if they are, the amounts are too small to detect. Apparently the change in conformation caused by the decreased temperature and/or the change of experimental conditions decreases the formation of the lysinolysine adduct.

A second side reaction that could occur is oxazoline formation observed by Fahrney and Gold on treatment of phenylmethanesulfonylchymotrypsin under acid conditions.[26] Some tosyl-labeled chymotrypsin was treated at pH 2 according to the procedure of Fahrney and Gold and then assayed by the pyruvic acid procedure. Only 0.2 mole of pyruvic acid was found. Since this value is the same as that found for native chymotrypsin,

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(39) B. S. Hartley, *Nature*, **201**, 1284 (1964).

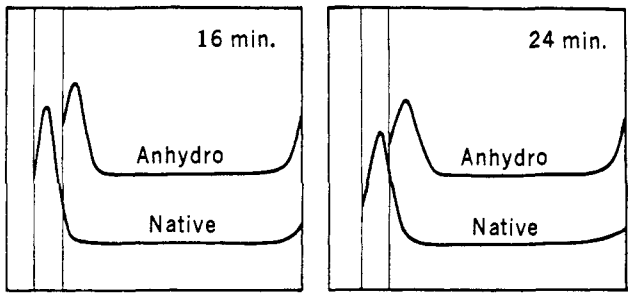


Figure 3. Ultracentrifuge patterns for native and anhydrochymotrypsin.

these results show that the pH 2 conditions do not produce dehydroalanine and that the oxazoline formation conditions cannot account for the dehydroalanine formed in the anhydro enzyme preparation. As a further test, the pyruvic acid assay was carried out on the tosyl enzyme and in this case 0.3 mole of pyruvic acid per mole of enzyme was obtained.

Amino acid analyses were also performed on various chymotrypsin preparations and are listed in Table V. No significant decrease in either the 10 cysteine residues or the 20 serine residues is observed as the result of the base treatment, and this is consistent with the pyruvic acid assay results.

Table V. Amino Acid Analyses of Chymotrypsin Preparations

Amino acid residue	Amino acid residues/25,000 g of protein: Native enzyme	Base-treated enzyme	Anhydro enzyme
Asp	22.9	22.4	22.5
Thr	20.8	20.9	20.9
Ser	25.0	25.7	24.7
Glu	16.5	16.6	16.9
Pro	10.6	11.1	10.1
Gly	24.3	23.9	24.4
Ala	22.5	22.2	22.5
Val	22.9	22.8	23.5
Met	1.8	2.1	2.0
Ileu	9.6	9.7	9.8
Leu	19.0	19.7	18.7
Tyr	3.6	3.9	3.6
Phe	6.0	6.1	6.1
Lys	13.1	14.4	14.1
His	2.1	2.2	2.2
Cysteic[a]	9.8	10.6	10.7

[a] From performic oxidized samples.

Taken together the results of many approaches indicate the absence of any major side reaction caused by the base treatment. The major share, therefore, of the reaction that does occur involves the conversion of serine at the active site to dehydroalanine.

Assays for Enzyme Activity. Assays were performed on the various chymotrypsin preparations and the results are recorded in Table VI. It is seen that base treatment of native enzyme causes a slight decrease in activity (approximately 20%) using the ATEE assay, but apparently all the molecular species are active since there is no decrease in the all-or-none assay. The base treatment of the native enzyme must therefore increase K_M or decrease V_M slightly without causing any loss of essential function. The modification reflected by the production of 0.4 additional mole of pyruvic acid appears, therefore, not to affect the catalytic site or conformation in any vital way.

Table VI. Assays on Chymotrypsin Preparations

Enzyme preparation	Relative ATEE assay,[a] %	All-or-none assay,[b] %
Native	100	100
Base treated	80	100
Anhydro	<0.01	<0.01
Tosyl	<0.1	<0.1

[a] Assay of Schwert and Takenaka.[30] [b] Assay by tosyl fluoride method[22] or by cinnamoylimidazole method.[21]

The enzymatic activity of the base-treated native enzyme is in contrast to the lack of activity of base-treated tosylchymotrypsin. The anhydro enzyme produced by the latter reaction is inert to both assays. Further experiments to observe activity by more accurate analytical conditions were attempted and under no condition could activity be observed. To test the possibility that the specificity of the enzyme had been changed[33] the anhydro enzyme was incubated with acetyltyrosinamide, but again no activity was detectable. A limit of approximately 0.01% might have been detected by these various methods and therefore a decrease of at least 10^4 in the reactivity of the protein has been caused by the conversion of the serine residue to dehydroalanine.

Physical Measurements on Anhydrochymotrypsin. Ultracentrifuge experiments on native, base-treated, and anhydrochymotrypsin were performed. In Figure 3 the ultracentrifuge patterns for native and anhydro enzyme are shown. The sedimentation velocity as measured by the position of the peak of the sedimenting protein is the same for these two proteins (1.905 for anhydro *vs.* 1.945 for native). The added width of the anhydro peak indicates added heterogeneity in this preparation. This is consistent with formation of dehydroalanine at positions other than the active site. Since the small amount of dehydroalanine formed at positions other than the active site has a small effect on the activity of the enzyme, it is reasonable that it would make only a small change in the sedimentation velocity. Thus, the ultracentifuge results indicate that the anhydro enzyme has the same state of aggregation as the native enzyme and probably essentially the same conformation.

Similar conclusions can be drawn from optical rotatory dispersion data.[40] Both native enzyme and anhydrochymotrypsin gave good agreement with the Drude equation and the respective constants were for λ, 246 and 236 mμ, respectively, and for a, -236 and -248, respectively. Cotton troughs of about the same magnitude were also observed for both enzymes at 233 mμ. Thus, the optical rotatory dispersion data also indicate small changes in conformation.[40]

It was noted that the anhydro enzyme was appreciably less soluble than the native enzyme. For example, the lyophilized preparation appeared to have a maximum solubility of 8 mg/ml at pH 7. (At pH 3 it was more soluble.) This might indicate a conformation change but might also indicate a change in mutal interactions caused by the deletion of the hydrophilic hydroxyl group of the serine.

(40) H. Weiner and D. E. Koshland, Jr., *J. Mol. Biol.*, **12**, 881 (1965).

Reaction with TPCK. Schoellman and Shaw[24] have demonstrated that the chloromethyl ketone derived from phenylalanine (TPCK) reacts with a single histidine at the active site of chymotrypsin. Since the removal of the serine hydroxyl had caused complete loss of activity in relation to ATEE, it was of interest to test the selectivity of this inhibition. The results are shown in Table VII.

Table VII. Histidine Reactivity in Chymotrypsin Preparations Treated with TPCK[a]

Chymotrypsin preparation	Treatment with TPCK Time, hr	pH	Histidine residues/ molecule of protein
Native enzyme (no TPCK)	..	...	2.1
Native enzyme	24	6.0	1.2
Native enzyme	48	6.0	1.1
Base-treated chymotrypsin	24	6.0	1.1
Anhydrochymotrypsin	24	6.0	2.1
Anhydrochymotrypsin	24	6.0	1.9
Anhydrochymotrypsin	48	6.0	2.0
Anhydrochymotrypsin	48	7.0	2.1
Tosylchymotrypsin	24	6.0	1.9
Tosylchymotrypsin	48	6.0	1.9

[a] A solution of N-tosyl-L-phenylalanyl chloromethyl ketone (2 ml, 3.4×10^{-3} *M*) in methanol was added to 100 ml of 0.10 *M* phosphate buffer at pH 6 or pH 7 containing 7.2 mg of protein preparation. After incubating for 24 or 48 hr at 25°, the samples were removed from the thermostat and acidified to pH 3 by addition of glacial acetic acid and then dialyzed against 10^{-3} *M* HCl. The dialysates were lyophilized and analyzed for amino acid content after acid hydrolysis.

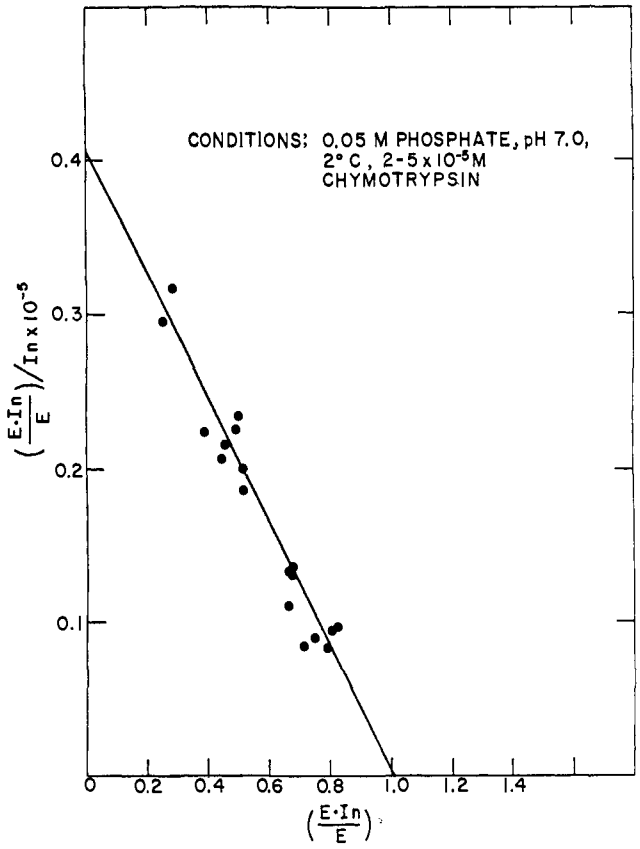


Figure 4. Plot of equilibrium dialysis data for binding of proflavine to chymotrypsin. Intercept on *x* axis indicates moles of proflavine bound per mole of chymotrypsin.

It is seen that control experiments with native enzyme or base-treated enzyme showed reaction with 1 mole of histidine. These results support previous deductions that the base treatment of the native enzyme does not cause any significant change in the catalytic features of the active site. No reaction was observed, however, between TPCK and anhydrochymotrypsin. Since DIP-chymotrypsin does not react with TPCK, the tosyl enzyme would also be expected to show no reaction with TPCK, and it did not.

Two alternatives appear possible in the interpretation of these results. One is that the activity of histidine is increased by serine just as it is supposed that histidine activates serine. A second involves the assumption that the steric relations of the active site have been altered by the conversion of serine to dehydroalanine. Since the Schoellman and Shaw reagent depends on the proper alignment of inhibitor at the active site, a change in the distance between the histidine and the specificity amino acids could cause the decreased histidine reactivity. It appears quite clear that the lack of reactivity is not caused by the failure to bind the TPCK since analogous substrates are found to retain an affinity for the enzyme surface (see following discussion).

Binding of Substrates to the Active Site. To test whether the change in the serine had affected the binding properties of the protein, equilibrium dialysis studies on the native and modified enzymes were performed. Because of possible dimerization of the native protein and the relative high K_M (10^{-3} *M*) of many of the substrates of chymotrypsin, a procedure based on the technique of Karush[29] in antibody studies was used. First the binding constant of an indicator molecule (proflavine) was ascertained. Proflavine appeared to be a valuable indicator molecule because of its high affinity as measured by Wallace, Kurtz, and Niemann[41] and its use in binding studies for chymotrypsin reported by Bernhard.[42] The displacement of this indicator molecule by high concentrations of substrate could be measured and the binding constant of the substrate could be determined even though the latter was not measurable by classical equilibrium dialysis procedures. The competitive dialysis procedures are especially valuable when it is desirable to screen a number of compounds without developing new assays in each case[29] or when the binding constants of inhibitors and the solubility of the enzyme preclude a classical equilibrium dialysis study.[3]

A Scatchard plot (Figure 4) indicates that 1 mole of proflavine is bound per mole of chymotrypsin and the slope of the line gives a binding constant of 2.5×10^{-5} *M*. From such plots the binding constants for proflavine with other protein preparations were obtained and are reported in Table VIII. The binding of proflavine to the native enzyme was also determined by competitive inhibition procedures using acetyltyrosine ethyl ester as a substrate. The value, 2.7×10^{-5} *M*, agreed with the analogous constant from equilibrium dialysis studies.

The association constant of proflavine to anhydrochymotrypsin is 12 times less than that of the native

(41) R. A. Wallace, A. N. Kurtz, and C. Niemann, *Biochemistry*, 2, 824 (1963).

(42) S. Bernhard and B. F. Lee, Abstracts, 6th International Congress of Biochemistry, 1964, Vol IV, p 297.

Table VIII. Dissociation Constants of Proflavine from Chymotrypsin Preparations[a]

Chymotrypsin preparation	Proflavine dissociation constant, $K_{IN} \times 10^5$ M
Native	2.5
Anhydro	29
Diisopropylphosphoryl	73
Tosyl	26
Chymotrypsinogen	67
TPCK treated	17

[a] pH 7.0, 0.05 M phosphate buffer, 2°, 2–10 $\times$ 10^{-5} M chymotrypsin preparation, 1–10 $\times$ 10^{-5} M proflavine.

enzyme but nevertheless the anhydro enzyme binds this indicator molecule more strongly than does diisopropylphosphoryl enzyme or chymotrypsinogen. In Table IX the binding of phenylpropionic acid and

Table IX. Dissociation Constants of Substrates for Native and Anhydrochymotrypsin[a]

Substrate	$K_S \times 10^3$ M for substrate Native chymotrypsin	Anhydro-chymotrypsin
β-Phenylpropionic acid	0.64 ± 0.02	5 ± 1
N-Benzoyl-L-phenylalanine	3 ± 1	7 ± 2

[a] pH 7.0, 0.05 M phosphate, 2°, 2–3 $\times$ 10^{-5} M enzyme, 2–4 $\times$ 10^{-5} M proflavine, 1–2 $\times$ 10^{-3} M substrate.

benzoyl-L-phenylalanine are shown derived from experiments with the competitive dialysis procedure. In these cases it is noted that the difference in binding constant between native and anhydro enzyme is less than in the case of proflavine, being a factor of 9 for the phenylpropionic acid and only a factor of 2 for the benzoyl-L-phenylalanine.

Discussion

The results reported above demonstrate (a) that a monosubstituted chymotrypsin with a single tosyl group on the serine at the active site can be prepared, (b) that the tosylchymotrypsin can be converted to anhydro enzyme by means of an elimination reaction in cold alkali, (c) that the conditions necessary for the conversion of the tosylated serine to dehydroalanine do not inactivate native chymotrypsin, (d) that the anhydrochymotrypsin is catalytically inert within the limits of experimental error, and (e) that the anhydrochymotrypsin has somewhat different binding properties from the native enzyme but that binding is changed in a minor way compared to the change in catalysis.

That a single tosyl group has been introduced is shown by the stoichiometry of the C^{14}-tosyl-labeled chymotrypsin. The conclusion that the serine at the active site has been tosylated is less obvious but is amply established by the derivatives produced by addition of such groups as sulfite, thioglycolic acid, etc., and by the almost quantative yields of pyruvic acid. In a sense this result was to be expected from the many studies of the activity of the serine at the active site and agrees with parallel studies of Fahrney and Gold.

The production of dehydroalanine by the elimination reaction is amply supported by chemical evidence. It is, therefore, of interest that Ginsberg and Wilson's study of model peptides found that tosylated serine peptides in an aqueous environment should produce oxazoline in preference to dehydroalanine. The fact that dehydroalanine is obtained under these conditions indicates that some special environmental factors exist in the protein. These factors might ultimately be of interest in clarifying the nature of the active site and may be explained when the three-dimensional structure of the protein is determined by X-ray crystallography.

The results further indicate that base treatment causes inactivation solely because of the conversion of the serine residue to dehydroalanine. Evidence for this conclusion comes from many quarters. In the first place amino acid analyses show no significant change in the molecule. Some serine and cysteine residues are probably converted to dehydroalanine residues but this conversion occurs in the "base-treated" enzyme as well as in the tosyl enzyme and does not lead to any loss of the all-or-none activity or to a change in the reactivity of the Schoellman and Shaw reagent. Some change in the ATEE "efficiency" assay is noted, *i.e.*, a 20% decrease, but clearly this is a minor perturbation in comparison with the complete loss of catalytic activity of the anhydro enzyme. The sedimentation studies, optical rotatory dispersion, and difference spectra all indicate that no major realignment of the protein occurs as a result of the treatment in alkali. In short all the extraneous and minor changes which occur as a result of alkali treatment occur on the conversion of native chymotrypsin to "base-treated" chymotrypsin as well as on the conversion of tosylchymotrypsin to anhydrochymotrypsin. Yet the base-treated enzyme is active. Therefore, the loss in activity must be caused by the change in the serine residue.

Since the dehydroalanine residue produced in the anhydro enzyme is smaller than the serine in the active protein, the steric access ambiguity present in previous modification studies is obviated. The loss in activity must result from some essential property that is changed by removing the elements of water from serine. It remains to be determined what essential property has been modified.

It has already been seen that no major change in the structure of the protein occurs, but a minor change in the geometry of the active site is compatible with the data. Such a small change could occur, for example, either because the hydroxyl group of the serine forms a key hydrogen bond maintaining a part of the structure of the active site or the change in the bond angle of the α-carbon atom from tetrahedral to planar might make subtle alterations in the three-dimensional relationships in the active site. That some such subtle alterations have occurred would be consistent with the slight change in the specificity of binding that is observed and in the failure of the TPCK to react with the histidine at the active site. The optical rotatory dispersion previously reported[40] also indicates a slight conformation change. Thus, it is possible that the serine modification has caused a change in the delicate orientation of the catalytic groups or of the relation between the specificity amino acids and the catalytic amino acids, and it is this disorientation rather than the loss of a direct catalytic

role of the serine residue which is responsible for the loss of activity in anhydrochymotrypsin.

Thus, the definitive identification of the precise role of the serine residue still eludes us. The number of alternative roles, however, have been further narrowed. It is seen that the serine residue cannot play any major role in the binding of substrate to the protein. Its modification may affect the specificity somewhat but the binding properties of the protein are changed only slightly from those of the native enzyme. The change in the catalytic properties is dramatic and whatever its detailed role the serine hydroxyl group must be intimately involved with the catalytic power of the enzyme.

The formation of anhydrochymotrypsin is perhaps the forerunner of the production of other proteins produced in an analogous way, *i.e.*, by converting an essential catalytic residue to a smaller residue. Such proteins which presumably will have lost their catalytic activity without having lost their binding properties may be useful in exploring the specificity relationships of enzymes in the absence of catalysis. Moreover, as in the case of chymotrypsin, a conversion of a residue to a smaller group eliminates arguments of steric access which would be important in any enzyme-catalyzed reaction especially if the active site is in a cleft of the molecule (as indicated for lysozyme)[43] where space is limited. Horecker and co-workers have utilized an elimination reaction in an ingenious way to remove complications of SH residues in studies on aldolase.[44]

A particularly useful property of the anhydro enzyme is that it offers the possibility of adding other atoms, for example, H_2S, to produce new proteins in which the catalytic residue has been converted to residues of similar appearance but with different chemical properties. From such "chemical mutations" the precise role of the serine residue may emerge.

Acknowledgment. The authors would like to express their gratitude for the invaluable assistance of John Ruscica during the course of this work. They would also like to acknowledge support from the National Institutes of Health (Grant AM 09765).

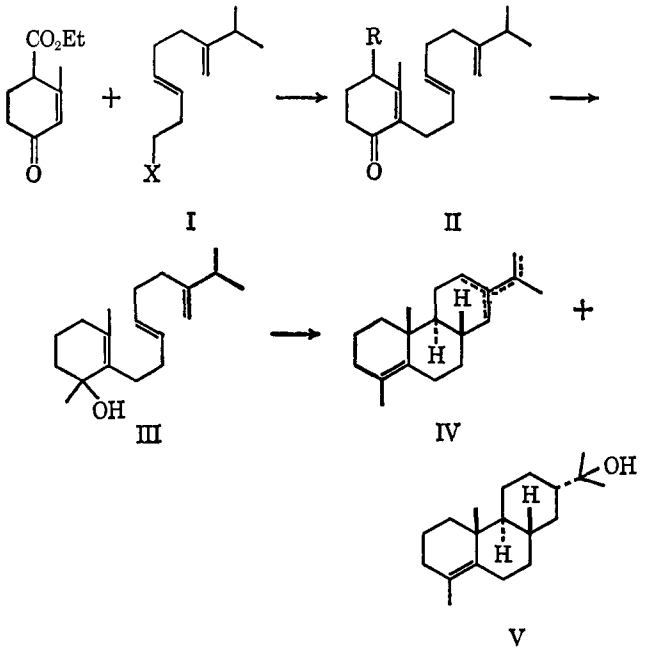
(43) C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and J. R. Sorma, *Nature*, **206**, 757 (1965).
(44) T. Cremona, J. Kowal, and B. L. Horecker, *Proc. Natl. Acad. Sci. U. S.*, **53**, 1395 (1965).

Communications to the Editor

An Efficient, Stereospecific Polyolefinic Cyclization. Total Synthesis of *dl*-Fichtelite

Sir:

In our search for polyolefinic systems that will undergo biogenetic-like polycyclization,[1] we have now extended previous ring-closure studies of butenylcyclohexenol systems[2] to include the cyclization of the trienol III. To our gratification we found, as described below, that this substrate undergoes stereospecific ring closure under mild conditions to give tricyclic material in very high yield. This finding has led to a facile total synthesis of *dl*-fichtelite (IX).



Following prior art[2b,c] we prepared the trienol III (*Anal.* Found: C, 82.2; H, 11.8) by alkylation of Hagemann's ester with the bromo diene I (X = Br) to produce II (R = CO_2Et). Hydrolysis and decarboxylation gave the unsaturated ketone II (R = H) which, on treatment with methyllithium,[2c] afforded III.

$$C_6H_5CH_2O(CH_2)_2C{\equiv}CCH_2X$$
VI

$$C_6H_5CH_2O(CH_2)_2C{\equiv}C(CH_2)_2COCH(CH_3)_2$$
VII

The bromide I (X = Br) was produced as follows. The product VI (X = OH) of reaction of the anion of the benzyl ether of 3-butyn-1-ol with formaldehyde was treated with phosphorus tribromide to give VI (X = Br). Reaction of VI (X = Br) with ethyl sodioisobutyrylacetate, followed by hydrolysis and decarboxylation, gave the ketone VII. Reaction of this ketone with methylenetriphenylphosphine, followed by treatment of the product with sodium in ammonia, gave the alcohol I (X = OH). The tosylate I (X = OTs), on

(1) W. S. Johnson, *Pure Appl. Chem.*, **7**, 317 (1963).

(2) (a) W. S. Johnson, W. H. Lunn, and K. Fitzi, *J. Am. Chem. Soc.*, **86**, 1972 (1964); (b) W. S. Johnson, P. J. Neustaedter, and K. K. Schmiegel, *ibid.*, **87**, 5148 (1965); (c) J. A. Marshall and N. Cohen, *ibid.*, **87**, 2773 (1965).