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*ROLE OF SERINE IN CHYMOTRYPSIN ACTION. CONVERSION OF THE ACTIVE SERINE TO DEHYDROALANINE**

BY D. H. STRUMEYER, WILLIAM N. WHITE, AND D. E. KOSHLAND, JR.

BIOLOGY DEPARTMENT, BROOKHAVEN NATIONAL LABORATORY

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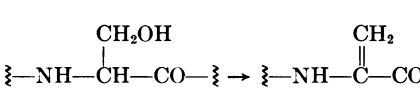
Serine is apparently *at* or *near* the active center of many enzymes. It is the residue that is phosphorylated or acylated by inhibitors and substrates in such enzymes as chymotrypsin,[1] trypsin,[1] thrombin,[2] elastase,[3] subtilisin,[4] cholinesterase,[5] phosphoglucomutase,[6] and alkaline phosphatase.[7] Although powerful arguments can be presented[8, 9] that it is indeed the key nucleophilic residue in the catalytic action of these enzymes, its role remains a source of controversy.

The reasons for this controversy are threefold. The first stems from the fact that essentially all of the "serine enzymes" have hydroxylic substrates, e.g., glucose and water. These acceptors have essentially the same reactivity as serine, and hence it is difficult to conceive of a catalytic role for a residue having the same properties as the final acceptor. The second objection stems from kinetic evidence[10–12] which is difficult to reconcile with an intermediate step involving serine; instead it suggests, or apparently requires, a direct attack by the water or glucose acceptor. Finally, all the reported modifications of serine which render an enzyme inactive involve replacing the H of the hydroxyl with a bulkier group. Hence, it can be argued that such serine modification prevents enzyme action because access to the true catalytic group, e.g., histidine, is blocked rather than because any intrinsic property of serine is changed.

To evaluate these arguments, a chymotrypsin in which the "active serine" residue was converted into a dehydroalanine residue (cf. equation 1) seemed highly desirable.



(1)

The conversion of the $-CH_2OH$ group to a $=CH_2$ group decreases the bulk of the side chain and hence eliminates the "steric access" argument. If the serine hydroxyl plays an essential role in the enzyme action, such a modified chymotrypsin should be inert. If the serine hydroxyl acts as a scavenger, being acylated in competition with the water, its absence should not prevent water reaction, and an active enzyme would be expected.

Several alternative routes to a dehydroalanine derivative were considered. Phosphate compounds can lead to dehydroalanine, but it has been established that more than one product results.[13] A tosyl derivative seemed more desirable since Photaki[14] has shown that tosyl derivatives of serine can be converted to dehydroalanine and Fahrney and Gold[15] have shown that phenylmethanesulfonyl fluoride reacts with the active serine of chymotrypsin. Tosyl chloride was used in our experiments and was found to give a preferential tosylation of the active serine.

Preparation of 'osyl-chymotrypsin: A solution of 500 mg of α-chymotrypsin in 50 ml of 0.2 *N* aqueous sodium chloride was kept at pH 7.0, while small portions of *p*-toluenesulfonyl-C^{14} chloride in dioxane were added at intervals. Time was allowed between individual additions for complete reaction to occur. The acid chloride additions were continued until the enzymatic activity had dropped to 5% of its original value as measured by the method of Schwert and Takenaka.[16]

The entire reaction mixture was subjected to gel filtration and the fractions corresponding to the peak in optical absorption (280 mμ) were combined and lyophilized to give 443 mg of fluffy white solid.

Radioassay of this material showed the presence of 0.99 toluenesulfonyl residues per molecule of chymotrypsin *p*-toluenesulfonate-C^{14}.

Kinetic study of the elimination of toluenesulfonic acid from tosyl-chymotrypsin: A solution of 40 mg of tosyl-chymotrypsin in 10.0 ml of water was cooled to 0° and treated with 10.0 ml of a cold 0.20 *N* sodium hydroxide solution with rapid mixing. The reaction mixture was kept at 0° and at various timed intervals 1 ml aliquots were added to 1.0 ml of cold 20% trichloroacetic acid solution to quench the reaction. From the measured radioactivities and the optical densities, it was possible to calculate the amount of radioactivity per mg of protein after the various timed intervals and thus the rate of elimination of the isotopically labeled toluenesulfonate group.

Preparation of anhydro-chymotrypsin: To an ice-cold solution of 300 mg of chymotrypsin *p*-toluenesulfonate-C^{14} in 25 ml of water was added rapidly, with swirling, 25 ml of ice-cold 0.20 *N* sodium hydroxide solution. After the mixture had stood in an ice bath for 4 hr, it was brought to pH 7.0 and subjected to gel filtration. The fractions corresponding to the peak in optical absorption (280 mμ) were combined and lyophilized to give 190 mg of fluffy white powder.

Radioassay of this material showed the presence of 0.22 toluenesulfonyl residues per molecule of anhydro-chymotrypsin. Essentially no loss and no gain in enzyme activity occurred during this detosylation procedure, i.e., the activity of the preparation was 5% after as well as before base treatment.

Base-treated chymotrypsin was prepared for control experiments by treatment of chymotrypsin exactly as described above for tosyl-chymotrypsin.

Results.—α-Chymotrypsin reacts readily with *p*-toluenesulfonyl chloride to form a tosylated derivative that has one toluenesulfonyl group per enzyme molecule. Several lines of reasoning led to the conclusion that this product is a sulfonic acid ester of the serine residue at the active site. First, it has been shown by Fahrney and Gold[15] that the analogous reaction of phenylmethanesulfonyl fluoride with chymotrypsin led to sulfonylation of the serine at the active site of the enzyme. Since both of these reactions led to inactivation of the enzyme, it appears that the same serine at the active site is tosylated in both cases. Secondly, the elimination experiments show that all but 10 per cent of the toluenesulfonyl group introduced into chymotrypsin via the sulfonyl chloride is easily removed by 0.1 *N* base at 0°.

Hydrolysis of sulfonamides (from lysine) and of sulfonic esters (from tyrosine) would not occur at an appreciable rate under these conditions, whereas an elimination reaction from the serine group[14] would have approximately this velocity. Finally, it should be noted that the group reacting with the sulfonyl chloride must have an unusual nucleophilicity, a property usually associated with the serine at the active site.

The tosyl-chymotrypsin has been shown by kinetic analysis to consist of three different substances. A plot of the extent of the elimination reaction versus time is shown in Figure 1, and it is clear that only 90 per cent of the toluenesulfonyl residue is eliminated. If the radioactivity remaining at 500 min is subtracted from earlier values, the corrected per cent losses shown in Figure 2 are obtained. The linearity of this log plot indicates that the majority of the protein molecules are present as a single species whose rate of elimination is indicated by the straight line. It is clear, however, that there is an initially rapid reacting material which is somewhat different from the bulk of the material present. The kinetic analysis indicates that about 12 per cent of the molecular species are in the especially reactive form, approximately 9 per cent in the inert form and 79 per cent in the form which eliminated at the velocity expected of a serine derivative. This analysis is further confirmed by a reaction with a tosyl-fluoride derivative which, from Fahrney and Gold's data, would be expected to be more selective for the serine residue and which was indeed found to react without producing any material which could not be eliminated by base.

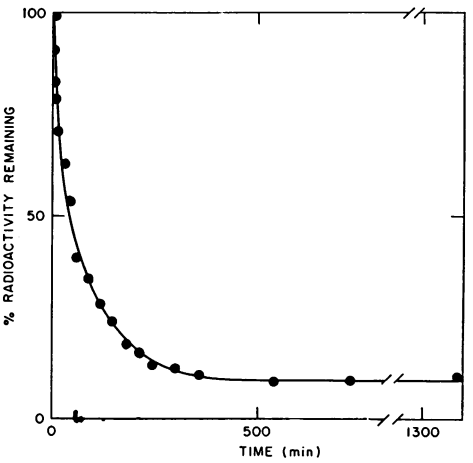


FIG. 1.—Elimination of the C^{14}-labeled tosyl group from tosyl-chymotrypsin on treatment with base.

The base treatment during the elimination reaction was shown not to result in denaturation of the enzyme. First, the small amount of enzyme activity (~5%) due to native chymotrypsin left in the tosyl-chymotrypsin preparation was virtually unchanged during the formation of anhydro-chymotrypsin. Second, similar base treatment of pure chymotrypsin resulted in a minor loss (less than 20%) of the enzymatic reactivity. Thus, the base treatment *per se* is not responsible for the presence of inactive enzyme. The inactivity of this modified chymotrypsin is therefore the result of the conversion of the serine residue to a dehydroalanine residue.

Despite the indirect evidence that the dehydroalanine had been prepared at the active site, it seemed desirable to demonstrate directly that such a residue was present. Therefore, several typical nucleophilic additions to an α,β-double bond were performed. The occurrence and extent of the addition reactions were determined by hydrolysis of the resulting protein and analysis for amino acid residues in the hydrolysate (cf. Table 1). The three addends used—sulfite ion, thioglycollic

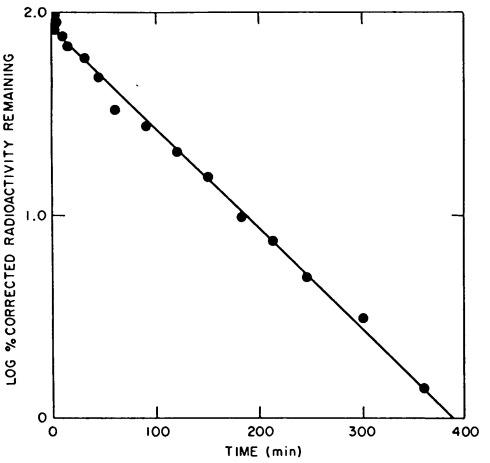


FIG. 2.—Elimination of C^{14}-labeled tosyl group from the active serine of tosyl-chymotrypsin on treatment with base. Data of Fig. 1 corrected for base-stable tosyl-chymotrypsin and plotted on logarithmic scale.

acid, and thiophenol—led to the formation of cysteic acid, carboxymethyl cysteine, and S-phenyl cysteine, respectively. Only small amounts of these products were formed when base-treated chymotrypsin was incubated with the adding reagents under the same conditions. These impurities may result from side reactions or perhaps an elimination reaction of the cysteine residues of the molecule. Thus, the finding that there were appreciably larger amounts of the adducts on treatment of anhydro-chymotrypsin as compared to base-treated chymotrypsin is corroborative evidence for the presence of a dehydroalanine residue in the anhydro-chymotrypsin.

Discussion.—The above experiments demonstrate that the conversion of the serine at the active site of chymotrypsin to dehydroalanine renders the molecule inactive. Since suitable controls establish that the treatments would not otherwise lead to inactivation, it is clear that it is the disappearance of the hydroxyl group which has led to the inactivation and hence that the hydroxyl group of serine plays a positive role in the action of chymotrypsin. The obvious role is that of a nucleophilic group at the active site. It might still be argued that the serine hydroxyl has a role in maintaining the tertiary structure of the active site, and this is a remote, but nevertheless finite, possibility. In any case, it is clear that the serine residue is not merely a scavenger which substitutes for the true acceptor, but is rather an essential group for the catalytic action of chymotrypsin.

Serine has also been implicated in the active center of a number of other esterases, in phosphoglucomutase, and in alkaline phosphatase. At times it has also been suggested that serine in these enzymes may play the part of a sink or scavenger rather than as a true catalytic group in the enzyme action. The evidence that serine plays a vital role in the catalysis of chymotrypsin therefore indicates that it probably plays a similar role in these other enzymes.

Finally, it is clear that the introduction of a double bond at the active site allows the possibility for the preparation of new types of derivatives of chymotrypsin. The adducts employed in the experiments described here were designed to demon-

TABLE 1

ADDITIONS TO THE DOUBLE BOND IN ANHYDRO-CHYMOTRYPSIN

Reagent to be added	Conditions	—Moles of Addition Product Produced— (Per Mole of Enzyme) Anhydro-chymotrypsin	Base-treated chymotrypsin
Na_2SO_3	20 hr, pH 9	0.56	0.21
Thioglycollic acid	24 hr, pH 8.2	0.95	0.55
Thiophenol	26 hr, pH 9.4	0.44	0.20

strate that such a double bond existed, but other adducts can be made whose properties may lead to a further understanding of the role of the serine residue in chymotrypsin. These studies will be published elsewhere.

Summary.—A modified chymotrypsin in which the serine residue at the active site has been transformed into a dehydroalanine residue has been prepared. This "anhydro-chymotrypsin" is inert. Since the serine modification in this case produces a smaller residue, the argument that serine modification prevents access to true catalytic residues is obviated. Therefore, it is concluded that serine cannot be a scavenger or alternate acceptor, but that the hydroxyl group of serine plays a direct positive role in the catalytic action of the enzyme.

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ON THE ASYMMETRY OF RNA SYNTHESIS IN VIVO

BY G. P. TOCCHINI-VALENTINI,* M. STODOLSKY,* A. AURISICCHIO,† M. SARNAT,* F. GRAZIOSI,† S. B. WEISS,‡ AND E. P. GEIDUSCHEK*

THE UNIVERSITY OF CHICAGO*,‡ AND INTERNATIONAL LABORATORY OF BIOPHYSICS AND GENETICS, NAPLES†

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The widely accepted thesis that RNA synthesis is required for the phenotypic expression of DNA-borne genetic information now has considerable experimental support.[1] Enzymes that catalyze RNA synthesis on a DNA template have also been isolated,[2–5] and these yield biosynthetic RNA capable of hybridizing to homologous DNA and of stimulating amino acid incorporation *in vitro*,[6, 7] although no