*THE CONVERSION OF SERINE AT THE ACTIVE SITE OF SUBTILISIN TO CYSTEINE: A "CHEMICAL MUTATION"**

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Since the disruption of the three-dimensional structure of a protein can cause loss of enzyme activity, the interpretation of modification studies at the active sites of enzymes becomes particularly difficult. It is necessary to modify catalytic residues to establish their essentiality to the catalytic process, but any modification leading to loss in activity might also be explained as an effect on three-dimensional structure. Moreover, it is not clear how much change in the spatial position of functional groups can be tolerated by the enzyme. Although some change is apparently allowed in residues involved in binding or tertiary structure,[1-3] it would be expected that enzyme activity should be even more sensitive to changes in amino acids directly involved in catalysis. Therefore, it would be desirable to modify the catalytic residues in as minor a way as possible in order to understand the function of these residues.

It has been possible to transform the serine residue at the active site of chymotrypsin to a dehydroalanine residue, a change which converted the serine to a smaller residue and thus removed any steric access ambiguities in the interpretation of the role of the serine.[4, 5] Such a modification, however, involved a considerable change in the chemical properties of the affected moiety, and it was desirable to attempt a conversion to a residue more nearly similar to that of the original serine. Such a residue is cysteine since the SH group of cysteine possesses steric and chemical properties similar to those of the OH group of serine. The van der Waals radii of oxygen and sulfur are 1.40 and 1.85 Å, respectively, and the covalent radii are 0.66 and 1.04 Å, respectively.[6] Since the two groups react similarly in hydrolyses, such a chemical transformation would be minimal, both chemically and sterically. The SH group is, in general, somewhat more reactive than the OH group either as an attacking nucleophile or as a leaving group.[7-10]

To effect such a change in chymotrypsin is difficult because the chemical manipulations require the presence of active sulfide compounds which tend to break the disulfide bridges in the molecule. Therefore, subtilisin, a proteolytic enzyme with properties like those of chymotrypsin but containing no disulfide bridges, was chosen for the desired conversion. The serine at the active site of subtilisin undergoes inactivation with diisopropylfluorophosphate,[11] and the specificity of the enzyme is similar in many ways to that of chymotrypsin and other serine proteases.[12] Chymotrypsin shows unusual reactivity with sulfonyl fluorides[13] and this reactivity was also found to extend to subtilisin. With the use of C^{14}-labeled phenylmethanesulfonyl fluoride, a rapid and stoichiometric inhibition of the enzyme was obtained yielding 0.95 ± 0.05 mole of phenylmethanesulfonyl group per mole of subtilisin. Apparently the phenylmethanesulfonyl group reacted by a prior binding to the active site, just as in the case of chymotrypsin, since the reactivity of the similar reagent, toluene sulfonyl fluoride, was much lower with both enzymes.

It seemed probable that this phenylmethanesulfonyl group in subtilisin could be

displaced by various sulfur anions since such displacements had been observed in model compounds[14, 15] and in chymotrypsin.[4] Both sulfide ion and thiolacetate ion were capable of stoichiometrically displacing the C^{14}-phenylmethanesulfonyl group and producing the conversion to a cysteine residue. The initial product of thiolacetate displacement is probably acetyl thiol-subtilisin, but since the thiolacetyl group is readily hydrolyzed either in small model compounds or in enzymes, the acetyl group on the thiol enzyme probably also hydrolyzes to yield free mercaptan. The presence of cysteine in the modified protein was confirmed by the presence of cysteic acid after performic acid oxidation, by the presence of carboxymethyl cysteine after reaction with iodoacetate, and by titration of the thiol enzyme with p-chloromercuribenzoate or the colored sulfhydryl reagent, 5,5′-dithio *bis*-(2-nitrobenzoic acid). Yields ranged from 0.1 to 1.3 moles per mole of protein depending upon the exact conditions used. The preparations used for further studies contained about 0.7 mole of cysteine as determined by amino acid analysis following performic acid oxidation.

The following reaction conditions gave good yields. Eleven μmoles of subtilisin in 20 ml, pH 7, 0.1 *M* phosphate buffer, and 10^{-4} *M* $CaCl_2$ were reacted for 2 hr with 15 μmoles of phenylmethanesulfonyl fluoride. The esterolytic activity against either acetyl tyrosine ethyl ester or p-nitrophenyl acetate dropped to less than 1 per cent of the original activity. The solution was then either made 0.7 *M* with potassium thiolacetate (Eastman) and adjusted to pH 5.5 *or* made 1.0 *M* with a solution of Na_2S which had been adjusted to pH 7.3. After 2 days at room temperature the solution was passed through G-25 Sephadex and lyophilized. The yield of protein was generally about 50 per cent of the original weight. Novo subtilisin[12] gave consistently higher conversion to thiol enzyme than the subtilisin BPN′, and thiolacetate at pH 5.5 appeared to give greater conversion than hydrogen sulfide anion at pH 7.

A small amount of regeneration to native enzyme activity apparently occurs during the displacement reaction since some activity (less than 2–3%) in the final protein is not inhibited by p-chloromercuribenzoate. Protein from control experiments in which native subtilisin was treated with thiolacetate or sulfide ion contained no cysteine. Further evidence that the serine to cysteine modification occurs at the active site is the fact that the displacement of the phenylmethanesulfonyl group to form cysteine could be partially inhibited by the presence of a typical competitive inhibitor, N-acetyl-L-phenylalanine.

After the hydroxyl group was converted to a sulfhydryl group, the activity of the modified enzyme was tested against a variety of substrates to determine any changes in activity. The comparison of activities of a typical preparation of the native Novo enzyme with the analogous thiol-subtilisin is shown in Table 1. In each case the activity which is not inhibited by the p-chloromercuribenzoate is subtracted from the total activity. Although the p-chloromercuribenzoate noninhibitable activity (probably representing regenerated native enzyme) is a very small quantity, it does place a limit on the ratios of activities which can be calculated within the limits of experimental error.

It is seen that the activity of the thiol enzyme toward nitrophenyl acetate is an appreciable percentage (33%) of the activity of the native enzyme toward this same reagent. Against other ester and peptide substrates of subtilisin no activity has

TABLE 1

COMPARISON OF THE ACTIVITY OF SUBTILISIN AND THIOL-SUBTILISIN

Substrate	Initial rate (ΔA/min/μg) Subtilisin	Thiol-subtilisin	Relative rate (%) 100× thiol-subtilisin/ subtilisin
NPA[a]	9.0×10^{-4}	3.0×10^{-4}	33
ATEE[b]	2.8×10^{-3}	$<6 \times 10^{-6}$ (footnote *c*)	<0.2
TAME[d]	7.0×10^{-5}	$<1 \times 10^{-6}$	<1.4
GPNA[e]	1.4×10^{-6}	$<7 \times 10^{-9}$ (footnote *f*)	<0.5
Ovalbumin[g]	2.6×10^{-1}	~0[f, h]	0
Casein[g]	4.0×10^{-1}	~0[f, h]	0

Hydrolysis of NPA, ATEE, TAME, and GPNA was measured by the change in absorbance (ΔA) at 407, 237, 247, and 385 mμ, respectively, in 0.05 *M* phosphate or 0.1 *M* Tris buffer. Hydrolysis of heat-denatured ovalbumin and casein was measured by reacting aliquots taken at various times with ninhydrin and determining the absorbance at 570 mμ.

a 1×10^{-3} *M* p-nitrophenyl acetate, pH 7.0.

b 2×10^{-3} *M* N-acetyl-L-tyrosine ethyl ester, pH 8.0.

c A small amount (0.5%) of native enzyme activity was regenerated which was not inhibitable by p-chloromercuribenzoate. This value was subtracted from the total activity to produce the quoted value.

d 2.5×10^{-3} *M* N-toluenesulfonyl-L-arginine methyl ester, pH 7.7.

e 1.5×10^{-3} *M* N-glutaryl-L-phenylalanine p-nitroanilide, pH 8.0.

f Native enzyme activity was 2–3% in this case. See footnote *c*.

g 10 mg/ml, pH 8.0.

h No activity was detectable under the conditions used.

been detected for the thiol enzyme. Acetyl tyrosine ethyl ester, tosylarginine methyl ester, glutarylphenylalanine p-nitroanilide, and the proteins albumin and casein, which present many peptide bonds susceptible to subtilisin action, were not significantly hydrolyzed by the thiol-subtilisin. Other peptides, carbobenzoxy phenylalanyl leucine and carbobenzoxy tryptophyl serine, were not substrates for native subtilisin and showed no hydrolysis with thiol-subtilisin.

Ratios of at least 100 in relative activities were observed but the activity of the thiol enzyme may be as much as 1,000- or 10,000-fold less than the native serine enzyme. The presence of mercaptoethanol did not change this reactivity so the loss of activity was not due to oxidation of the thiol group during assay. Experiments in the pH range of 5–9 have been performed with the same results. Raising or lowering the substrate concentration (acetyl tyrosine ethyl ester) did not affect the observed rate of hydrolysis by the thiol enzyme, which indicates that an increase in K_m or substrate inhibition does not account for the decreased rate. Indeed, the apparent K_m of nitrophenyl acetate with thiol subtilisin (8×10^{-5} *M*) is *lower* than that found with the native enzyme (5.6×10^{-4} *M*).

It seems unlikely that the loss of activity for the specific substrates is caused by a disruption of the active site due to the fairly harsh treatment with thiolacetate. Parallel treatment of the subtilisin which had not been phenylmethanesulfonylated resulted in only 20 per cent loss of activity toward acetyl tyrosine ethyl ester and no loss of activity toward nitrophenyl acetate. The loss of activity of the native enzyme during the incubation with thiolacetate was probably largely due to autolysis. It is logical to assume that this loss represents the maximum loss since the phenylmethanesulfonyl enzyme could not undergo any such autolysis. Preliminary experiments indicate that the binding of acetyl tyrosine ethyl ester and the ultracentrifugal pattern of the thiol enzyme are essentially the same as the native enzyme. The possibility that a nonspecific unfolding of the protein occurs during modification seems unlikely in view of the retention of activity by the control treated under similar conditions.

The activity of the thiol-subtilisin toward nitrophenyl acetate at first may seem

TABLE 2

RATE OF HYDROLYSIS OF NITROPHENYL ACETATE BY THIOL-SUBTILISIN AND BY GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

	pH 7.0 (0.05 *M* phosphate)	pH 8.5 (0.05 *M* Tris)
Thiol-subtilisin	0.015 sec^{-1}	0.025 sec^{-1}
Glyceraldehyde phosphate dehydrogenase	0.0007 sec^{-1}	0.12 sec^{-1}
Ratio: Thiol-subtilisin/glyceraldehyde phosphate dehydrogenase	21	0.21

The steady-state rate of appearance of nitrophenolate ion, as measured by the changes in absorbance at 407 mμ, was determined at a substrate concentration of 8×10^{-4} *M*, a saturating level. The observed rate was corrected for the spontaneous hydrolysis of nitrophenyl acetate and normalized to the number of active sites present per mole. DPN-free glyceraldehyde phosphate dehydrogenase was used.[18]

at variance with the lack of activity toward specific substrates. Yet the comparison of the reaction of nitrophenyl acetate with other mercaptans renders this activity less surprising. Nitrophenyl acetate was found to react rapidly with free cysteine in solution.[16, 17] It reacts readily with thiol groups in proteins such as glyceraldehyde phosphate dehydrogenase,[18] an enzyme which is not designed for reaction with nitrophenyl acetate. At pH 7, thiol-subtilisin hydrolyzes nitrophenyl acetate more rapidly than does glyceraldehyde phosphate dehydrogenase, but at pH 8.5 this ratio is reversed since the rate of the glyceraldehyde phosphate dehydrogenase reaction increases sharply in this region (cf. Table 2 and ref. 18). Since nitrophenyl acetate is an activated ester whose hydrolysis is catalyzed by many nucleophiles including cysteine, it is probable that an SH group on any protein will show some reactivity with this reagent. Preliminary studies of the kinetics of the reaction in our laboratory and more extensive studies on a similar protein by Polgar and Bender[19] indicate that the nitrophenyl acetate reaction with thiol-subtilisin has some of the features of an enzymatic reaction, i.e., preliminary complex formation prior to the hydrolytic reaction. It is therefore possible that the activity toward nitrophenyl acetate indicates that the protein is still acting in a catalytic manner and that the exploration of these properties will be useful in delineating the role of the SH group or other groups in the enzyme. It is also possible, however, that the new protein is completely inert in the normal enzymatic sense and that the reactivity of the sulfhydryl group toward nitrophenyl acetate only reflects a minor perturbation in the environment of this group when it is placed on the surface of a protein. Analogous changes in the reactivities of histidines,[20] methionines,[3] and other functional groups are observed when they are present in proteins.

The somewhat surprising lack of reactivity of the thiol enzyme toward the more normal substrates of subtilisin indicates that the conversion of the serine to cysteine has dramatically changed the properties of the protein. From the study of model reactions one would expect a thiol group to be more reactive than a hydroxyl group. Thus, in the nucleophilic attack on aryl halides the RS^- anion is 50–4,000 times more reactive than the RO^- anion.[7] Toward p-nitrophenyl acetate the thiophenoxide ion is more reactive than the phenoxide ion by 30-fold[17, 21] and the mercaptoethanol anion is also more reactive than phenoxide ion.[8] Moreover, at neutral pH the lower pK of the thiol group makes it possible to have far more mercaptide anion than alcoholic anion in equivalent concentrations of base. Even with highly reactive compounds such as mustard gas cation[9] or acetyl imidazole,[10] the thiol group is more reactive than the analogous oxygen compound under equivalent circumstances.

As leaving groups, thiols are far better than the equivalent oxygen compounds. For example, OH^- catalyzes the hydrolysis of thiol esters and oxygen esters about equally well[22, 23] but the thiol esters are much more susceptible to nitrogen nucleophiles.[22, 24] Only in the case of acid hydrolysis of esters are the thio derivatives less reactive, in this case by factors of $^1/_{10}$ to $^1/_{30}$.[25] Thus, the change of a serine residue to a cysteine residue would involve a change in size of the functional group by approximately 0.40 Å, a small change in bond angle, a change in reactivity probably in the direction of a more reactive intermediate (on the basis of model compounds), and a change to a more polarizable atom.

Studies of inductive effects in substrates and acylated enzymes and the pH dependence of catalysis strongly suggest that a nucleophilic reaction occurs during the action of chymotrypsin and similar proteases.[26] The sulfhydryl enzyme would be expected to be more reactive both in the initial nucleophilic attack on the substrate to form an acyl enzyme and in the subsequent deacylation in which the cysteine enzyme acts as a leaving group. If this is indeed the mechanism in the two steps of the reaction, then the lack of reactivity in this case must be ascribed to the change in geometry at the active site, i.e., the increase in radius of 0.4 Å and the change in bond angle due to the sulfur atom. It is indeed surprising that such a small change in size could produce such dramatic results on the activity of the enzyme.

It may be noted that thiol esters are less susceptible to acid hydrolysis than are oxygen esters and it is conceivable that a general acid-catalyzed deacylation of the enzyme might become critically rate-determining in the case of thiol-subtilisin. However, even here the thiol esters in model compounds are only moderately less reactive (10–30 times)[25] than the oxygen esters so that a great enhancement of the acid effect must occur if this is to explain the lack of reactivity of the thiol enzyme. A possibility that the polarizability of the sulfur is responsible must also be considered although *a priori* this change would be expected to increase the rate.

The physicochemical properties of this enzyme should be of particular interest because of the potentialities for exploring the influence on the active site of even minor changes in the catalytic groups. Thus, the effect of the new thiol group on the reactivity and position of other important side chains should be a fruitful area of research.

Genetic mutations have been extremely useful in providing modified proteins for correlation of structure and function, e.g., tobacco mosaic virus protein[27] and tryptophan synthetase.[1] However, most mutations in nature are limited to the 20 amino acids in proteins and therefore, except for the serine-cysteine conversion, any change in the residues which take part in catalysis involve relatively large modifications of charge and size. The results presented here suggest that genetic mutations of catalytic residues will almost invariably produce inactive proteins. The fact that most amino acid mutations do not produce inert protein would therefore reflect the fact that only a very small percentage of the residues of any one protein are directly involved in catalysis. Since inactive enzymes are not easy to isolate from mutants and since most interchanges of natural amino acids produce gross structural changes, "chemical mutations" in the laboratory provide a reasonable alternative for the subtle modification of active site amino acids.

While this work was in progress, the authors learned of a similar study by Polgar

and Bender.[19] Their work has emphasized the reactivity of thiol-subtilisin toward nitrophenyl esters, whereas this paper emphasizes the lack of activity of thiol-subtilisin toward specific ester and peptide substrates. Continuing work on this enzyme should provide additional information for the correlation of structure and function of enzymes.

Summary.—The hydroxyl of the serine in the active center of subtilisin has been converted to a sulfhydryl group. The resulting enzyme, thiol-subtilisin, is at most $^1/_{100}$ as active as native subtilisin toward normal ester and peptide substrates. Since model experiments indicated that the thiol group should in general be more reactive than a hydroxyl group, the loss of activity of thiol-subtilisin indicates the enormous sensitivity of catalytic residues to subtle changes in structure.

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