221, 61.
Yoshida, A. (1965a), *J. Biol. Chem. 240*, 1113.
Yoshida, A. (1965b), *J. Biol. Chem. 240*, 1118.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

The Reactivity of Thiol-subtilisin, an Enzyme Containing a Synthetic Functional Group*

Laszlo Polgar† and Myron L. Bender

ABSTRACT: A synthetic enzyme was prepared by transforming the reactive serine residue in the active site of subtilisin to a cysteine residue. The transformation was accomplished nearly quantitatively by treating the serine residue with phenylmethanesulfonyl fluoride (PMSF), displacing the PMS group with thiolacetate ion, and allowing the acetyl-thiol-subtilisin to deacylate enzymatically. Titration with *p*-mercuribenzoate (PMB) and amino acid analysis indicated the presence of thiol groups and cysteine residues, respectively. Thiol-subtilisin catalyzes the hydrolysis of *p*-nitrophenyl acetate. This reaction, which is inhibited by *p*-mercuribenzoate, is characterized by a presteady-state burst of *p*-nitrophenol followed by a zero-order, steady-state release of *p*-nitrophenol. The steady-state reaction obeys Michaelis–Menten kinetics. Comparison of *p*-nitrophenyl acetate hydrolyses catalyzed by subtilisin and thiol-subtilisin indicates identical k_{cat}/K_m, differing k_{cat} and K_m, similar pK values of k_{cat}, and different pK values of k_{cat}/K_m. The decinnamoylation of subtilisin and thiol-subtilisin derivatives shows similar pK values but the former is much faster than the latter. *trans*-Cinnamoyl-thiol-subtilisin, however, is much more sensitive toward nucleophiles like glycinamide than is the counterpart of the native enzyme. The hydrolyses of acylamino acid esters and amides are not sensibly catalyzed by thiol-subtilisin. Thiol-subtilisin catalyzes certain hydrolytic reactions by a mechanism similar to that of native subtilisin with the exception that the acyl-enzyme is a thiol ester.

The reactions catalyzed by hydrolytic enzymes usually take place through the formation of an intermediate acyl-enzyme. In the most studied hydrolytic enzymes, the hydroxyl group of a serine or the thiol group of a cysteine residue forms an intermediary covalent bond with the substrate. Since these two amino acid residues differ only in an oxygen or sulfur atom of the side chain, it is an interesting problem in enzymology to transform the two types of hydrolytic enzymes into one another. Such a transformation might contribute to a better understanding of the mechanism if the artificial enzyme retains activity.

To perform the transformation, one should find a reaction taking place specifically at the active site of the enzyme. Specific reactions with a complex molecule like a protein are not always feasible but have been demonstrated in a few instances (*e.g.*, Balls and Jansen, 1952; Schoellmann and Shaw, 1963; Lawson and Schramm, 1965).

Recently investigations on small peptides demonstrated the conversion of a serine to a cysteine residue with retention of optical activity (Photaki and Bardakos, 1965; Zioudrou *et al.*, 1965). The reaction was performed in three steps: (1) the reaction of the serine residue with tosyl chloride; (2) the displacement of the sulfonyl compound by thiol acetate ion; (3) the hydrolysis of the thiol ester formed in step 2. This reaction series may be applied to an enzyme if it contains no disulfide bridges, to avoid damaging reactions with thiolacetate through disulfide interchange. Such a hydrolytic enzyme is subtilisin. A preliminary report on the transformation of subtilisin to thiol-subtilisin was previously published (Polgar and Bender, 1966). Here we report some of the characteristics of this synthetic enzyme.

Experimental Section

Subtilisin, Bacterial Proteinase Novo, was purchased from the Novo Pharmaceutical Co., and Nagarse from Teikoku Chemical Industry Co., Ltd. *p*-Nitrophenyl acetate was recrystallized from alcohol–water and melted at 114.5°. *N-trans*-Cinnamoylimidazole, a product of the Aldrich Chemical Co., Inc., was recrystallized four times from dry hexane, mp 134.0°.

* From the Division of Biochemistry, Department of Chemistry, Northwestern University, Evanston, Illinois 60201. *Received October 24, 1966.* This research was supported by grants from the National Institutes of Health.

† On leave from the Institute of Biochemistry of the Hungarian Academy of Sciences, Budapest, Hungary.

PMSF[1] and PMSF-1′-^{14}C was purchased from Calbiochem, Inc.; thiolacetic acid potassium salt from the Eastman Kodak Co.; and PMB from Mann Research Laboratories, Inc.

The concentration of subtilisin was measured spectrophotometrically using the data of Matsubara *et al.* (1965), $A_{278}^{1\%}$ 11.7, mol wt 27,600. The same data were employed with thiol-subtilisin. The thiol- and PMS-group content of the enzyme always refer to this determination.

The sulfhydryl content of the protein was titrated with PMB at pH 7 according to Boyer (1954). The absorption of the S–Hg bond with thiol-subtilisin was assumed to have the same extinction coefficient as Boyer found with cysteine, $\Delta\epsilon$ 7.6 $\times$ 10^3 at 250 mμ. Radioactivity was measured on a Tri-Carb liquid scintillation counter using 15 ml of Bray's mixture (1960) and 1.0 ml of the sample in water.

Amino Acid Analysis. Before hydrolysis, the enzyme was gel filtered through a Sephadex G-25 column in distilled water at 5°. The protein fraction was dried in a vacuum desiccator over phosphorus pentoxide. Protein (10 mg) was dissolved in 1 ml of formic acid, and 1 ml of performic acid (a mixture of formic acid (four parts) and hydrogen peroxide (one part) incubated 15 min at 5°) was added and incubated at 5° for 30 min. Then the mixture was evaporated in a flash evaporator at room temperature, the residue was dissolved in water and reevaporated, and the latter procedure was repeated. The oxidized protein was dissolved in 2 ml of redistilled hydrochloric acid, sealed under vacuum, and hydrolyzed at 107° for 24 hr. After removing the hydrochloric acid, the hydrolysate was applied to a Spinco amino acid analyzer.

Preparation of PMS-1′-^{14}C-subtilisin. Subtilisin (0.143 g) was dissolved in 5 ml of 0.1 M phosphate buffer, pH 7, and incubated with 1.23 mg of PMSF-1′-^{14}C in 0.1 ml of dioxane (0.35 mc/mmole) for 10 min at 25°. The reaction mixture was gel filtered through a Sephadex G-25 column equilibrated with 0.05 M phosphate buffer, pH 6.2. The protein fraction contained 0.90 equiv of PMS group/mole of subtilisin as determined by the ratio of liquid scintillation counting to the absorption of gel-filtered solution.

Preparation of Thiol-subtilisin. Subtilisin (100 mg) was dissolved in 4 ml of 0.1 M phosphate buffer, pH 7.0, and incubated with 0.1 ml of PMSF solution (8.8 mg/ml of dioxane) at room temperature for 10 min. Potassium thiolacetate (400 mg) was added and the pH of the solution was adjusted to 5.25 with 0.2 mM hydrochloric acid and kept for 72 hr at room temperature. The reaction mixture was then gel filtered on a Sephadex G-25 column equilibrated with 0.05 M phosphate buffer, pH 7.0. When thiol-subtilisin was used for the preparation of *trans*-cinnamoyl-thiol-subtilisin, gel filtration was performed in 0.1 M acetate buffer, pH 5.0. Different batches of thiol-subtilisin prepared in this way (from both the Novo and Nagarse enzymes) contained 0.85–0.98 equiv of sulfhydryl group as determined by PMB titration. The enzyme was kept at 0° under nitrogen. Slow inactivation, presumably by oxidation of the sulfhydryl group, occurred.

Preparation of trans-Cinnamoyl-subtilisin. Subtilisin (30 mg) was dissolved in 1.0 ml of 0.1 M acetate buffer, pH 5.0, and incubated with 1.0 mg of *N-trans*-cinnamoylimidazole dissolved in 0.1 ml of acetonitrile for 1 min. The reaction mixture was gel filtered through a Sephadex G-25 column equilibrated with 0.1 M acetate buffer, pH 4.0. The protein fraction contained the *trans*-cinnamoyl-subtilisin. *trans*-Cinnamoyl-thiol-subtilisin was prepared in the same way as *trans*-cinnamoyl-subtilisin, except the reaction mixture was about threefold diluted and gel filtration was performed at pH 5.0.

Kinetic Measurements. Reactions were measured on a Cary 14 recording spectrophotometer equipped with a thermostated cell compartment at 25.0 ± 0.2°. *p*-Nitrophenyl acetate hydrolysis was measured according to Kézdy and Bender (1962). The reaction was corrected for spontaneous hydrolysis. In the case of thiol-subtilisin $k_{cat}/K_m = k_2/K_s$ was determined directly from first- and second-order reactions as described by Kézdy and Bender (1962). The first-order rate constant k_{exp}[1] equals $(k_2/K_s)E_0$ if either $K_s > E_0 > S_0 > K_m$ or $E_0 < S_0 < K_m$. The second-order rate constant k_{exp}[2] equals k_2/K_s if $K_s > E_0 \approx S_0 > K_m$. Decinnamoylation of subtilisin and thiol-subtilisin derivatives was followed at 310 and 320 mμ, respectively.

Results

The Reaction of Subtilisin with Phenylmethanesulfonyl Fluoride. In order to transform the reactive hydroxyl group of subtilisin to a thiol group, the serine residue must first be treated with a sulfonyl compound. Tosyl chloride used with small peptides (Photaki and Bardakos, 1965; Zioudrou *et al.*, 1965) did not prove to be a suitable reagent. Its reaction with the enzyme was very sluggish. We found, however, that PMSF reacts rapidly and stoichiometrically even at high dilution (2 $\times$ 10^{-4} M PMSF inactivates about 99% of subtilisin in 10 min using *p*-nitrophenyl acetate as substrate). Using radioactive PMSF we found that 0.9 equiv of inhibitor can be bound to Novo Subtilisin, while, concurrently, the enzymatic activity drops below 1% as tested with *p*-nitrophenyl acetate as substrate. This implies that the enzyme preparation (after gel filtration) contains 90% active subtilisin and approximately 10% inactive protein, probably partly denatured enzyme.

The binding of the PMS group to subtilisin offers a method for the titration of the active site, *i.e.*, for the determination of the concentration of the active enzyme. By means of kinetic measurements we attempted to determine whether the fast reaction between the enzyme and the inhibitor is a result of the binding of PMSF to subtilisin, *i.e.*, whether the enzyme can be saturated with the inhibitor. The first-order rate constant of the

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; PMB, *p*-mercuribenzoate.

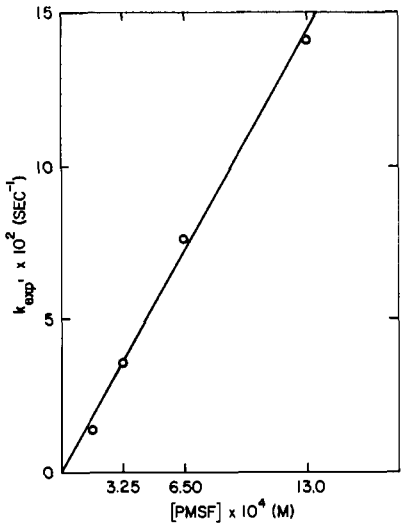

FIGURE 1: Inactivation of subtilisin by PMSF. *p*-Nitrophenyl acetate (1.78×10^{-5} M), acetonitrile (1.8%, v/v), and dioxane (1.8%, v/v) in 0.1 M phosphate buffer, pH 7.95. The ratio of the concentrations of PMSF and subtilisin was 65. The reaction was initiated by the addition of enzyme.

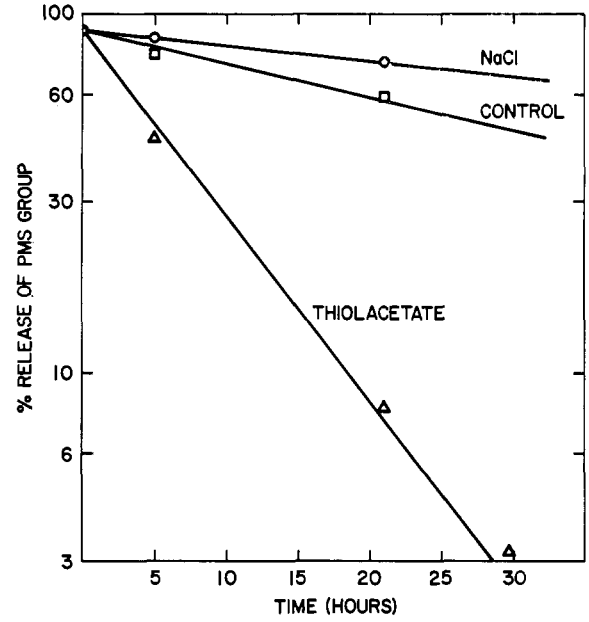

FIGURE 2: The release of PMS group of PMS-subtilisin at pH 5.22. Subtilisin (6×10^{-5} M) containing 0.9 equiv of radioactive PMS group was incubated in 0.1 M acetate buffer, pH 5.22, at 25°. PMS-subtilisin was also incubated in the presence of 0.7 M sodium chloride and 0.7 M potassium thiolacetate, respectively. Aliquots of the reaction mixtures were gel filtered on Sephadex G-25 columns equilibrated with 0.1 M phosphate buffer, pH 7.0, and the radioactivity of the protein fractions was determined.

inactivation of the enzyme was calculated from the decreasing rate of the *p*-nitrophenyl acetate hydrolysis catalyzed by subtilisin in the presence of PMSF. If the substrate (*p*-nitrophenyl acetate) and the inhibitor (PMSF) concentrations are lower than their Michaelis constants and do not change during the reaction, that is, $S = S_0 < K$ and $I = I_0 < K$, the first-order inhibition rate constant k_{exp}^1 can be determined from the liberation of the nitrophenol (see Appendix). The rate of inactivation is $-dE/dt = k_I I_0 E$, where E is the active subtilisin concentration and $k_{exp}^1 = k_I I_0$. Figure 1 shows k_{exp}^1 plotted *vs.* I_0. The linear increase of k_{exp}^1 with I_0 indicates no saturation by the inhibitor up to 1.3×10^{-3} M PMSF concentration, *i.e.*, $I_0 < K_m^I$.

The Reaction of Thiolacetate Ion with PMS-subtilisin. The displacement of the sulfonyl group of the tosyl peptides was accomplished in a mixture of organic solvent and phosphate buffer at pH 7.0 (Photaki and Bardakos, 1965; Zioudrou *et al.*, 1965). The neutral medium is favorable for working with proteins, but we could not remove the PMS group from the enzyme under these conditions even if we added dimethylformamide to the reaction mixture up to 40% (v/v). At this concentration of organic solvent, the enzymatic activity is not impaired significantly and the reaction conditions are almost the same as those used for the transformation of the serine to cysteine peptides.

Possibly at neutrality the reactive carbon atom of the serine residue of the PMS-subtilisin cannot react with the thiolacetate ion either because of steric or electrostatic hindrance. Therefore, we tested the reaction of the sulfonyl enzyme with thiolacetate ion at lower pH, which might change the steric structure of the protein and at the same time provide it with positive charge. At pH 4.4, PMS-subtilisin easily loses its sulfonyl group even without thiolacetate ion. This phenomenon may be similar to that found for PMS-chymotrypsin (Gold and Fahrney, 1964). At pH 5.22, the release of PMS groups is significantly lower. Figure 2 shows that high ionic strength (0.7 M NaCl) stabilizes PMS-subtilisin against loss of the sulfonyl group. Thus, the high concentration of thiolacetate not only promotes the formation of acetyl-thiol-enzyme but also decreases the proton-catalyzed release of the PMS group.

The Preparation and Analysis of Thiol-subtilisin. The experiment shown in Figure 2 indicates that the PMS-subtilisin should be transformed to acetyl-thiol-subtilisin in good yield. In fact, PMS-^{14}C-subtilisin, when incubated at pH 5.2–5.3 with 0.7 M thiolacetate ion for 48–72 hr at 25°, releases more than 99% of its radioactivity. From the reaction mixture the thiol enzyme can be isolated by gel filtration through a Sephadex G-25 column equilibrated with 0.05 M phosphate buffer, pH 7.0. At this pH the acetyl-thiol-subtilisin, formed from the reaction with thiolacetate ion, should deacetylate if the enzyme preserves any catalytic activity. Hence, the thiol group should be

titratable with PMB. After isolation of the thiol enzyme from the reaction mixture by gel filtration and incubation at pH 7.0 and 25° for 30 min, 0.85–0.90 equiv of thiol group was titrated in the enzyme which previously contained 0.90 equiv of the PMS group. The enzyme concentration was determined by measuring the absorption at 278 mμ (see Experimental Section). In different batches of subtilisin up to 0.98 equiv of thiol group/mole of enzyme was titrated. These analyses indicate the practically quantitative conversion of the subtilisin to thiol-subtilisin under the conditions described.

As control experiments we titrated both subtilisin and subtilisin incubated with thiolacetate under the conditions employed for the preparation of thiol-subtilisin, but less than 0.1 equiv of thiol group was found in both cases. This indicates that thiolacetate ion reacts only with the PMS-serine residue of the inhibited enzyme and thus the thiol group of the thiol-subtilisin exists in a cysteine residue.

To obtain direct evidence for the existence of cysteine in thiol-subtilisin, we determined a portion of the neutral and acidic amino acids of subtilisin and thiol-subtilisin. The proteins were oxidized with performic acid to transform the cysteine to cysteic acid, a more convenient species for analysis.

Table I shows that our analysis is in good agreement with that of Matsubara *et al.* (1965). Furthermore, while the oxidized subtilisin contains cysteic acid only as an impurity, almost the expected amount of cysteic acid was found in the thiol-subtilisin after performic acid oxidation. These data offer evidence that the transformation of the serine to cysteine residue has taken place.

TABLE I: Amino Acid Analysis of Subtilisin and Thiol-subtilisin.

Amino Acid	Number of Amino Acid Residues per Mole of: Subtilisin[a]	Subtilisin	Thiol-subtilisin Sample 1	Thiol-subtilisin Sample 2
Glu	16	15.9	16.1	16.2
Val	15	14.8	14.7	15.2
Cys-SO_3H	—	0.096	0.76	0.80

[a] Matsubara *et al.* (1965).

The Hydrolysis of p-Nitrophenyl Acetate Catalyzed by Thiol-subtilisin. *p*-Nitrophenyl acetate proved to be a suitable substrate to study the enzymatic activity of thiol-subtilisin. The hydrolysis of *p*-nitrophenyl acetate catalyzed by the thiol-enzyme is inhibited by PMB to the extent of 95–99%, while this inhibitor does not reduce the enzymatic activity of native subtilisin at all. This implies that the thiol-enzyme is sufficiently pure for use in these kinetic investigations.

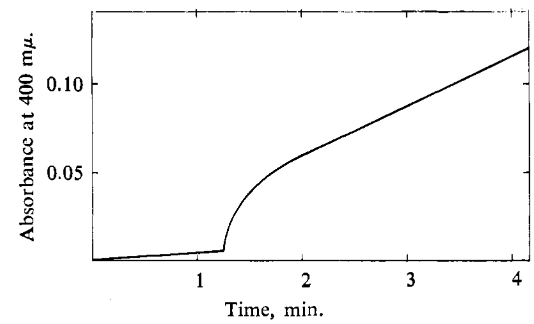

FIGURE 3: Hydrolysis of *p*-nitrophenyl acetate (1.1×10^{-4} M) catalyzed by thiol-subtilisin; 0.1 M phosphate buffer containing 1.8% (v/v) acetonitrile–water at pH 7.95.

The kinetics of the thiol-subtilisin-catalyzed hydrolysis of *p*-nitrophenyl acetate *is* similar to that of chymotrypsin, subtilisin, and papain and presumably takes place according to eq 1

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} E\cdot S \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (1)$$
$$+\ P_1$$

where the symbols have their usual meaning (Kézdy and Bender, 1962).

In a presteady-state reaction, a burst of nitrophenol was observed corresponding to rapid acetyl-enzyme formation. The burst was followed by a steady-state (zero-order) reaction, indicating a rate-determining deacylation (Figure 3).

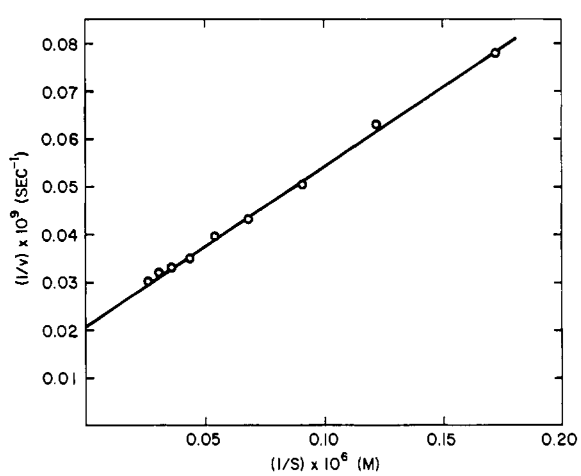

FIGURE 4: A Lineweaver–Burk plot of the steady-state reaction of the thiol-subtilisin-catalyzed hydrolysis of *p*-nitrophenyl acetate at 25.0° in 1.8% (v/v) acetonitrile–water, pH 7.84, 0.1 M phosphate buffer. $E_0 = 6.2 \times 10^{-6}$ M, $S_0 = 51.5 \times 10^{-6}$ M.

TABLE II: Kinetic Constants of Hydrolysis of *p*-Nitrophenyl Acetate Catalyzed by Subtilisin.[a]

pH	Buffer (0.1 M)	E_0 (10^4 M)	S_0 (10^4 M)	k_{cat} (sec^{-1})	K_m (10^4 M)	$1/K_m$ (10^{-4} M^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})
5.89	Phosphate	0.197	7.4	0.0127	4.70	0.213	27
6.45	Phosphate	0.348	14.5	0.0405	4.60	0.218	88
6.97	Phosphate	0.348	14.5	0.096	3.88	0.258	250
6.99	Phosphate	0.197	7.4	0.0925	3.40	0.296	270
7.78	Phosphate	0.348	14.5	0.240	4.40	0.228	550
7.80	Phosphate	0.255	14.5	0.258	5.10	0.196	510
7.84	Phosphate	0.197	7.4	0.231	3.82	0.262	610
7.91	Veronal	0.467	14.5	0.248	5.08	0.197	490
8.36	Veronal	0.467	14.5	0.294	5.30	0.187	560
8.65	Veronal	0.467	14.5	0.298	4.40	0.228	680
9.35	Carbonate	0.467	14.5	0.298	4.35	0.230	680
9.43	Carbonate	0.348	14.5	0.293	4.40	0.228	670
9.82	Carbonate	0.467	14.5	0.305	5.00	0.200	610

[a] 25.0°, 1.8% (v/v) acetonitrile–water.

TABLE III: Kinetic Constants of the Hydrolysis of *p*-Nitrophenyl Acetate Catalyzed by Thiol-subtilisin.[a]

pH	Buffer (0.1 M)	E_0 (10^6 M)	S_0 (10^6 M)	k_{cat} (10^3 sec^{-1})	K_m (10^6 M)	$1/K_m$ (10^6 M^{-1})
5.95	Phosphate	2.71	22.2	0.51	1.95	0.512
6.48	Phosphate	2.55	22.3	2.00	4.25	0.235
6.50	Phosphate	2.20	22.0	1.25	4.16	0.240
6.99	Phosphate	2.71	22.2	3.80	6.60	0.152
7.00	Phosphate	2.55	22.3	4.35	8.40	0.119
7.25	Veronal	6.20	51.5	4.85	11.7	0.086
7.45	Veronal	5.00	51.0	7.00	13.8	0.072
7.52	Phosphate	3.90	65.5	6.15	13.2	0.076
7.84	Phosphate	6.2	51.5	8.42	17.2	0.058
7.87	Phosphate	7.6	103.0	6.90	18.3	0.055
7.93	Phosphate	2.71	66.6	7.38	15.2	0.066
8.00	Veronal	5.00	51.0	8.50	20.0	0.050
8.04	Veronal	5.85	51.0	9.05	15.8	0.063
8.41	Veronal	6.20	51.5	9.20	21.7	0.046
8.43	Veronal	5.60	51.0	8.10	21.8	0.046
8.48	Veronal	5.00	51.0	8.70	19.6	0.051
8.57	Veronal	5.85	51.0	9.50	17.8	0.056
8.70	Veronal	5.00	51.0	8.50	20.9	0.048
9.29	Carbonate	5.85	51.0	10.0	22.2	0.045
9.51	Carbonate	6.90	51.5	10.0	33.0	0.030

[a] 25.0°, 1.8% (v/v) acetonitrile–water.

The steady-state portion of the hydrolysis obeys Michaelis–Menten kinetics. A Lineweaver–Burk plot (1934) is shown in Figure 4.

The kinetic parameters, k_{cat} and K_m, were calculated from Lineweaver–Burk plots. Tables II and III and Figure 5 demonstrate the pH dependence of k_{cat} in subtilisin and thiol-subtilisin-catalyzed reactions, respectively. Assuming dependence on a single ionizable group, k_{cat} (limit) and pK values can be calculated from these curves according to L. J. Brubacher and F. J. Kézdy (unpublished data, 1965).

Table IV shows that the pK values of both enzyme

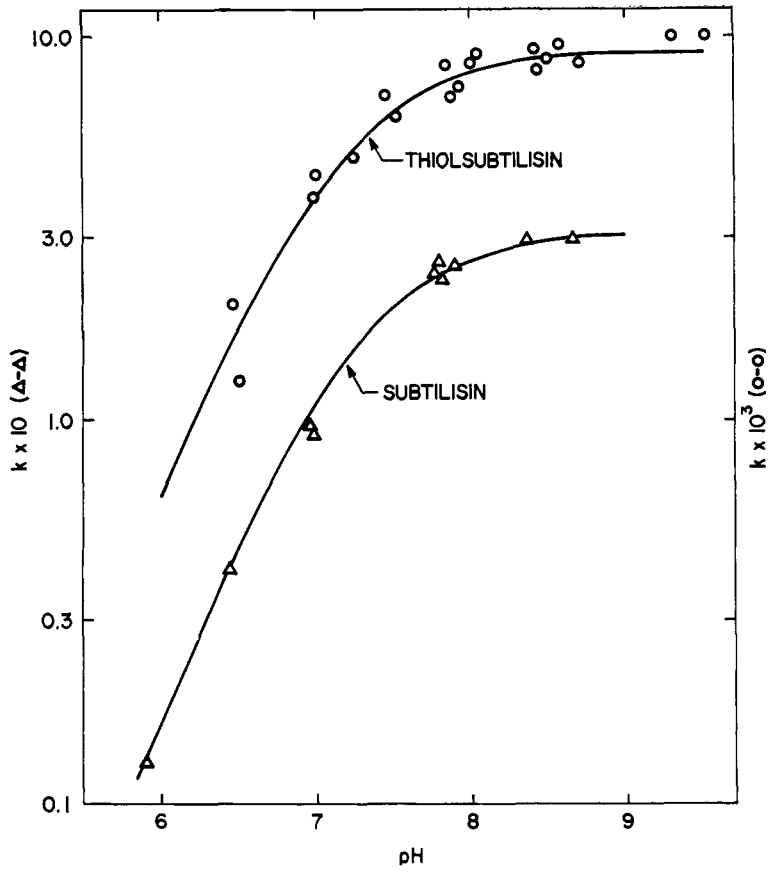

FIGURE 5: pH dependence of k_{cat} of *p*-nitrophenyl acetate hydrolysis catalyzed by subtilisin and thiol-subtilisin at 25°. k_{cat} values were calculated from Lineweaver–Burk plots (data of Tables II and III). The points are experimental. The curve is theoretical (see text).

reactions are similar, while k_{cat} (limit) decreases more than 30-fold in the transformation of subtilisin to thiol-subtilisin. These results suggest that in both subtilisin and its thiol derivative the same histidine residue participates in the deacylation, though the rate of the deacylation is significantly lower in acetyl-thiol-subtilisin.

The pH–K_m profiles of the two enzymes are entirely different. As can be seen in Figure 6, the K_m of subtilisin does not change with pH, while the K_m of thiol-subtilisin strongly decreases ($1/K_m$ increases) at lower pH values.

Figure 7 and Table V illustrate the pH dependence of k_{cat}/K_m of subtilisin and thiol-subtilisin reactions, respectively. The curve for subtilisin was determined by dividing k_{cat} by K_m while for thiol-subtilisin the data were calculated directly from first- and second-order kinetics as described in the Experimental Section. Table IV shows that k_{cat}/K_m (limit) is the same with both enzymes within experimental error. The profiles, however, are significantly different. The pK of k_{cat}/K_m for subtilisin is similar to that found in the pH–k_{cat} profile, but there is no measurable pK value between pH 5.5 and 9.5 for the k_{cat}/K_m of thiol-subtilisin. More data at lower pH could not be determined because of the instability of the protein, while the spontaneous

TABLE IV: Kinetic Constants of the Hydrolysis of *p*-Nitrophenyl Acetate.[a]

Enzyme	k_{cat} (limit) (sec^{-1})	pK of k_{cat}	k_{cat}/K_m (limit) (M^{-1} sec^{-1})	pK of k_{cat}/K_m
Subtilisin	$3.1 \pm 0.2 \times 10^{-1}$	7.27 ± 0.10	680 ± 90	7.30 ± 0.15
Thiol-subtilisin	$9.3 \pm 0.7 \times 10^{-3}$	7.15 ± 0.15	570 ± 60	—

[a] 25.0°, 1.8% (v/v) acetonitrile–water.

TABLE V: k_2/K_s of *p*-Nitrophenyl Acetate Hydrolysis Catalyzed by Thiol-subtilisin.[a]

pH	Buffer	Kinetic Order	k_2/K_s (M^{-1} sec^{-1})
5.20	Acetate	2nd[b]	305
5.30	Acetate	1st[c]	276
5.30	Acetate	1st[c]	365
5.85	Phosphate	2nd	480
5.91	Phosphate	2nd	538
5.94	Phosphate	1st[c]	440
6.06	Phosphate	1st[c]	584
6.14	Phosphate	2nd	590
6.92	Phosphate	2nd	590
7.01	Phosphate	1st[d]	660
7.01	Phosphate	1st[d]	536
7.94	Phosphate	1st[d]	476
7.95	Phosphate	1st[d]	525
7.96	Phosphate	1st[d]	592
7.97	Phosphate	1st[d]	522
8.00	Phosphate	1st[d]	486
8.88	Carbonate	1st[d]	400
9.25	Carbonate	1st[d]	360
9.46	Carbonate	1st[d]	365
9.86	Carbonate	1st[d]	360

[a] 25.0°, 1.8% (v/v) acetonitrile–water. [b] For second-order reactions, $E \simeq S > K_m$. [c] For these first-order reactions, $K_s > E > S > K_m$. [d] For these first-order reactions, $S < K_m$.

TABLE VI: Rate Constants of Deacylation of Cinnamoyl-Enzymes.[a]

pH	Buffer (0.1 M)	k_3 (sec^{-1})
	trans-Cinnamoyl-subtilisin	
6.95	Phosphate	0.0166
7.14	Veronal	0.024
7.29	Phosphate	0.033
7.91	Phosphate	0.088
8.45	Veronal	0.159
9.25	Carbonate	0.201
	trans-Cinnamoyl-thiol-subtilisin	
5.86	Phosphate	0.168[b]
6.45	Phosphate	0.373[b]
6.97	Phosphate	0.960[b]
7.26	Veronal	1.33[b]
7.98	Phosphate	3.45[b]
8.02	Phosphate	3.25[b]
8.09	Veronal	3.33[b]
8.42	Veronal	3.86[b]
8.67	Veronal	3.88[b]
8.95	Carbonate	4.23[b]
8.97	Carbonate	3.77[b]
9.11	Carbonate	4.10[b]
9.62	Carbonate	4.78[b]
10.49	Carbonate	9.50[b]

[a] 25.0°, 1.8% (v/v) acetonitrile–water. $E_0 \simeq 6 \times 10^{-6}$ M. [b] $\times 10^{-4}$.

hydrolysis of *p*-nitrophenyl acetate interfered with the determination at higher pH.

trans-Cinnamoyl-thiol-subtilisin. In a preliminary report we pointed out that thiol-subtilisin forms a cinnamoyl-enzyme with *N-trans*-cinnamoylimidazole and shows an absorption maximum at about 310 mμ which is characteristic of an *S*-cinnamoyl rather than an *O*-cinnamoyl bond (Polgar and Bender, 1966). *trans*-Cinnamoyl-subtilisin was also prepared, having an absorption maximum at 289 mμ, similar to that of *trans*-cinnamoyl-chymotrypsin but quite different from that of *trans*-cinnamoyl-thiol-subtilisin. Thus, the thiol-enzyme shows a perturbation of 4 mμ from that of the model *S*-cinnamoylcysteine, while the oxygen enzyme shows a perturbation of 7.5 mμ from that of the model *O*-cinnamoylserinamide (Bender and Brubacher, 1964). Both perturbations are to higher wavelength, but are somewhat smaller perturbations than seen with chymotrypsin, trypsin, or papain.

The preparation of *trans*-cinnamoyl-thiol-subtilisin offers the possibility of determining directly k_3, the rate constant of deacylation. Table VI and Figure 8 show the pH–k_3 profiles of the subtilisin and thiol-subtilisin deacylations, respectively. The k_3 (limit) and pK were calculated according to L. J. Brubacher and F. J. Kézdy (unpublished data, 1965) assuming dependence on one ionizable group; the data are compiled in Table VII. It can be seen that the pK values are higher in the deacylation (k_3) of the two cinnamoyl-enzymes than in the turnover (k_{cat}) of the two acetyl-enzymes; this difference is greater for native subtilisin than for thiol-subtilisin. The pK values for deacylation of different chymotrypsin derivatives also

TABLE VII: Kinetics of Decinnamoylation of Subtilisin and Thiol-subtilisin.[a]

Cinnamoyl-Enzyme	k_3 (limit)	pK	k_3 (limit) H_2O/k_3 (limit) D_2O
Subtilisin	$2.1 \pm 0.1 \times 10^{-1}$	8.05 ± 0.05	2.5[b]
Thiol-subtilisin	$4.3 \pm 0.3 \times 10^{-4}$	7.50 ± 0.15	2.86

[a] 25.0°, 1.8% (v/v) acetonitrile–water. [b] M. L. Begue (unpublished data, 1965).

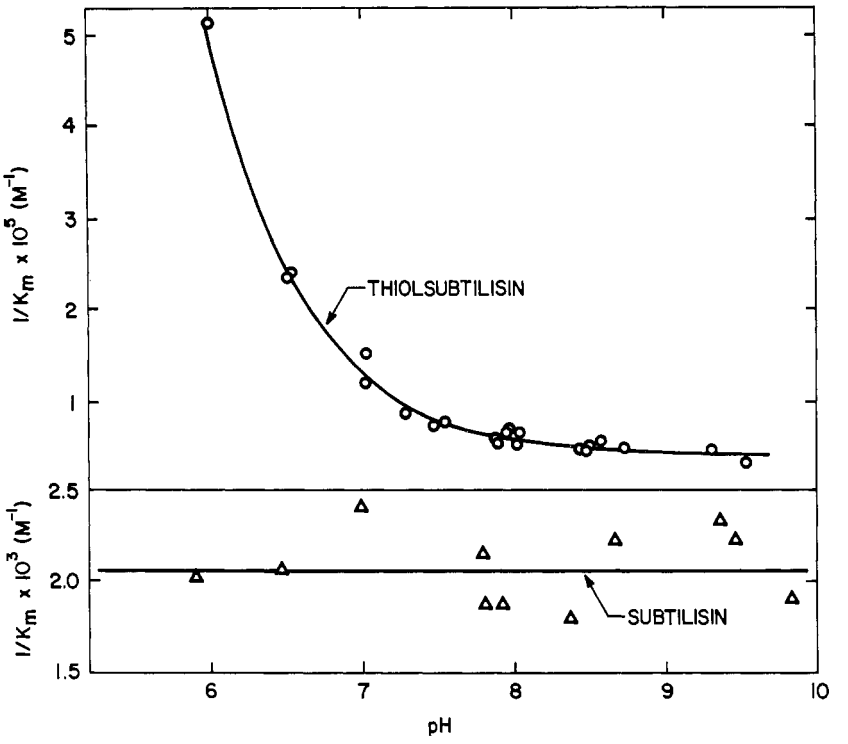

FIGURE 6: pH dependence of K_m of *p*-nitrophenyl acetate hydrolysis catalyzed by subtilisin and thiol-subtilisin. K_m values were calculated from Lineweaver–Burk plots. Data of Tables II and III.

depend on the substrate (Bernhard *et al.*, 1966). The rate constant of deacylation of cinnamoyl-thiol-subtilisin is lower than that of the corresponding acetyl derivative. Furthermore, there is more than two orders of magnitude difference in the rate constants of decinnamoylation of subtilisin and thiol-subtilisin.

In spite of the low rate, the decinnamoylation of the thiol-subtilisin derivative is an enzymatic hydrolysis on the basis of its pH dependence. Above pH 9.7, however, the flat part of the curve of Figure 8 starts to rise again (not shown), probably because hydroxyl catalysis overcomes the enzymatic one. This is supported by Figure 9. In the pH range corresponding to maximum enzymatic activity the decinnamoylation does not depend on the ionic strength (buffer concentration) or slightly decreases; however, at pH 10.45 it significantly increases, probably because the high buffer concentration reduces the effect of charge at the active site of the protein, thereby facilitating the approach of the hydroxyl ion to the protein surface.

Further evidence on the enzymatic decinnamoylation is provided by the D_2O effect. The reactions catalyzed by general bases are slower by about threefold in D_2O than in water (Bender *et al.*, 1962). Table IV shows that the decrease in the rate of decinnamoylation of both subtilisin and its thiol derivative in D_2O is in good agreement with the expected value.

Thiol esters react more easily with nitrogen nucleophiles than the corresponding oxygen esters. It was shown by Brubacher and Bender (1966) that amines increase the deacylation of cinnamoyl-papain to a much greater extent than oxygen nucleophiles. Table VIII illustrates the same phenomenon for cinnamoyl-thiol-subtilisin. The nucleophile employed, glycinamide, is without measurable effect on cinnamoyl-subtilisin.

The cinnamoyl-thiol-enzyme, in the presence of nucleophiles, reacts with both water and the nucleophile, $k_{exp} = k_3W + k_4N$ (Brubacher and Bender,

TABLE VIII: The Effect of Glycinamide on the Deacylation of Cinnamoyl-thiol-subtilisin and Cinnamoyl-subtilisin.

	Cinnamoyl-Enzyme of	
	Subtilisin[b]	Thiol-subtilisin[c]
k_3 (sec^{-1})	2.0×10^{-1}	4.1×10^{-4}
k_3 in presence of 2×10^{-2} M glycinamide (sec^{-1})	2.0×10^{-1}	12.5×10^{-4}
k_3 (M^{-1} sec^{-1})	3.6×10^{-3}	7.3×10^{-6}
k_4 (M^{-1} sec^{-1})	<0.5[a]	4.0×10^{-2}

[a] Assuming the experimental error of 5% reflects reaction by glycinamide. [b] pH 9.25. [c] pH 9.30.

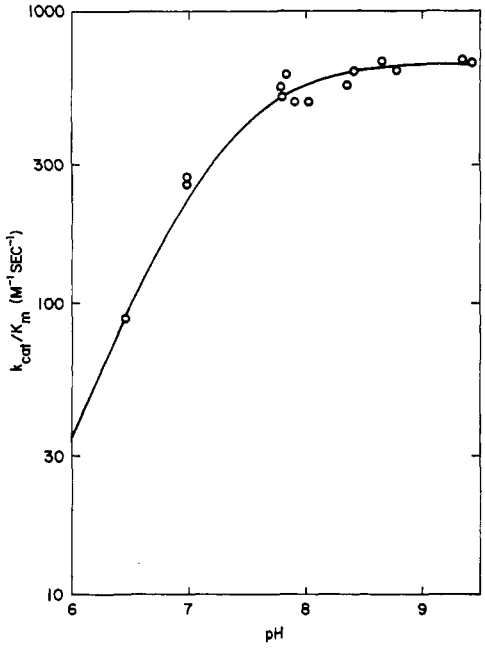

FIGURE 7: pH dependence of k_{cat}/K_m of *p*-nitrophenyl acetate hydrolysis catalyzed by subtilisin. Data of Table III.

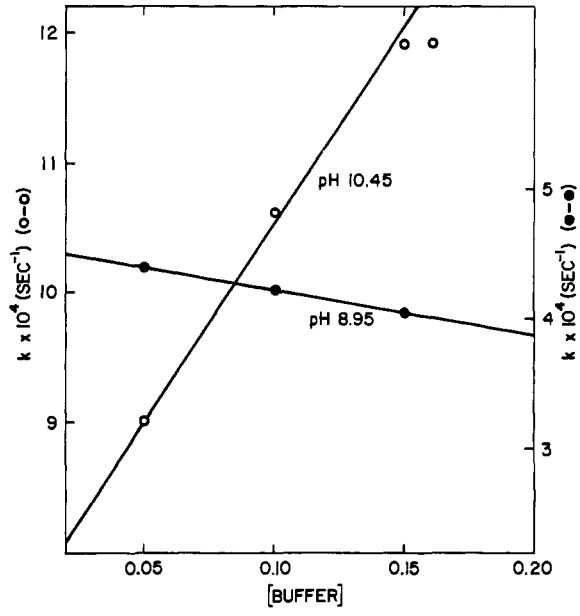

FIGURE 9: The effect of buffer concentration on the decinnamoylation of thiol-subtilisin. Reaction conditions were the same as in Figure 8. Carbonate buffers were used.

1966). Table VIII shows the rate constants of both the water and the glycinamide reactions. It can be seen that k_4 with thiol-subtilisin is about four orders of magnitude greater that k_3, whereas the (estimated) k_4 for subtilisin is less than two orders of magnitude greater than k_3.

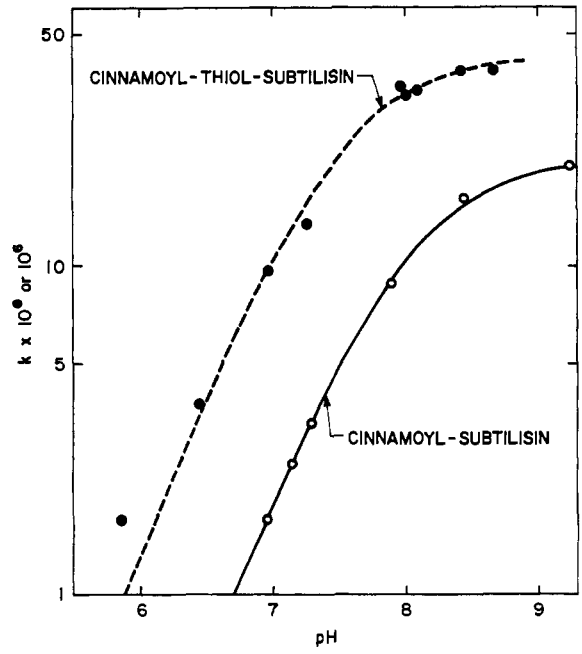

FIGURE 8: pH dependence of decinnamoylation of subtilisin and thiol-subtilisin. Decinnamoylation was measured at 25.0° and 310 mμ for thiol-subtilisin. Data of Table VI.

Discussion

This research was undertaken to prepare a synthetic enzyme by changing the reactive serine to a cysteine residue in the active site of subtilisin. This transformation was accomplished nearly quantitatively by treating the serine residue in the active site of subtilisin with PMSF, then displacing the PMS group with thiolacetate ion, and finally allowing the acetyl-thiol-subtilisin to deacylate enzymatically. The appearance of the thiol group in the protein was demonstrated by PMB titration and by determination of the difference spectrum of the cinnamoyl-enzyme (Polgar and Bender, 1966) which corresponds to an *S*-cinnamoyl rather than an *O*-cinnamoyl derivative. Amino acid analysis gave chemical evidence for the existence of cysteine in thiol-subtilisin.

The incubation of native subtilisin with thiolacetate ion does not produce thiol groups in the protein. Only subtilisin, previously treated with PMSF, is transformed to the thiol-enzyme in the presence of thiolacetate ion, the PMS group being released when the transformation takes place. These data offer evidence that the reactive serine in the active center was specifically replaced by a cysteine residue.

The catalytic activity of thiol-subtilisin toward *p*-nitrophenyl acetate and *N-trans*-cinnamoylimidazole substrates is significantly lower than that of subtilisin.

With alkyl esters and amide substrates, like *N*-acetyl-L-tryptophan methyl ester, *N*-acetyl-L-tryptophanamide, *N*-benzoyl-L-arginine ethyl ester, and *N*-benzoyl-L-argininamide, no activity inhibitable by PMB can be measured.[2] The inactivity of the thiol-enzyme toward these substrates may be due to either an inherent lower activity of the thiol-subtilisin which is obscured by the enzymatic activity of impurities, or may be due to a specificity of thiol-subtilisin only for good acylating substrates.

The second-order rate constant ($k_2/K_s = k_{cat}/K_m$) of thiol-subtilisin with *p*-nitrophenyl acetate at pH 6.9 is approximately 600 M^{-1} sec^{-1} (Table V), whereas the second-order rate constant for the reaction of cysteine with *p*-nitrophenyl acetate at pH 7 is 0.208 M^{-1} sec^{-1} (Whitaker, 1962). Although these figures really are not comparable since the thiol-subtilisin reaction is a catalysis whereas the cysteine reaction is not, the difference indicates that thiol-subtilisin is an enzyme of reasonable activity in relation to a non-enzymic reaction involving a similar nucleophile.

The reactivity of thiol-subtilisin toward substrates with good leaving groups appears to be similar to that found in the case of D-glyceraldehyde 3-phosphate dehydrogenase (Polgar, 1964). This thiol-enzyme, in addition to its physiological reaction, the oxidative phosphorylation of D-glyceraldehyde 3-phosphate, also hydrolyzes alcohol-activated esters possessing good leaving groups, but does not hydrolyze simple alkyl esters or acyl-activated esters. Since thiol-subtilisin hydrolyzes compounds such as *p*-nitrophenyl esters and *N-trans*-cinnamoylimidazole, possessing good leaving groups, the specificity of this enzyme is more like that of D-glyceraldehyde 3-phosphate dehydrogenase than that of papain, another thiol-enzyme which is able to hydrolyze not only *p*-nitrophenyl esters but also simple alkyl esters.

p-Nitrophenyl acetate was chosen as a substrate for thiol-subtilisin, since the enzymatic hydrolysis of this substrate could be almost completely inhibited by PMB; impurities do not complicate the kinetic analysis of the thiol-enzyme activity toward this substrate. The thiol-subtilisin-catalyzed hydrolysis of *p*-nitrophenyl *N*-benzyloxycarbonylglycinate was also investigated; however, its hydrolysis was only inhibited 60–70% with PMB; *i.e.*, some other enzymatic activity was associated with that of thiol-subtilisin. The impurity, though present in only a minor amount, has a greater specific activity than the thiol-enzyme toward *p*-nitrophenyl *N*-benzyloxycarbonylglycinate. The impurity is probably not subtilisin, because it was not inhibited by PMSF.

The k_{cat} of both subtilisin- and thiol-subtilisin-catalyzed reactions appears to depend on the same basic group with a pK about 7.2, which is presumably the imidazole group of a histidine residue. This implies that the histidine residue, participating in the catalytic activity of subtilisin, is able to assist the catalysis after the serine is changed to a cysteine residue. The absolute magnitude of k_{cat} which reflects the rate-limiting deacetylation is, however, significantly lower, probably because the larger sulfur atom changes the steric structure of the active site.

The pH dependence of k_{cat}/K_m of subtilisin is different from that of thiol-subtilisin. In subtilisin reactions a group with the same pK as that involved in the deacylation steps was found, but no group possessing a pK in the pH range 5.5–9.5 was associated with the thiol-enzyme reactions. One can calculate the pK of the catalytic group of the *acyl-enzyme* from the pH dependence of k_{cat} if deacylation is rate limiting. On the other hand, the pH dependence of k_{cat}/K_m is characteristic of the catalytic group of the *free enzyme* (Peller and Alberty, 1959).[3] Consequently free and acetyl-subtilisin, as well as acetyl-thiol-subtilisin, possess a group with a pK characteristic of a histidine residue, but free thiol-subtilisin does not. An attractive explanation concerning this exception is the existence of a hydrogen bond between the imidazole and the thiol groups of free thiol-subtilisin. This bond should stabilize both the imidazole and the thiol groups, which implies that the pK of the histidine group should decrease and the pK of the thiol group should increase. Model experiments show that hydrogen bond formation can change the pK 2–5 units (Bender *et al.*, 1963). If such hydrogen bond formation exists in thiol-subtilisin, the pK of the imidazole could be lowered and the pK of the thiol group could be raised so that both pK values would be outside the experimental range of pH 5.5–9.5. In subtilisin, however, either a different steric structure or the orientation of orbitals of the oxygen atom does not permit the formation of a hydrogen bond.

In spite of the presumed change in steric structure of the active site of subtilisin due to the transformation to thiol-enzyme, the synthetic enzyme retains enzymatic activity, though lower than that of its native counterpart. In some aspects, like deacylation, the mechanism of action of thiol-subtilisin resembles that of the native enzyme. There is a significant difference, however, in acylation. The lower pK of acylation of thiol-subtilisin compared to that of subtilisin results in a much higher rate of acylation by *p*-nitrophenyl acetate with the thiol derivative than with native subtilisin at low pH values.

Appendix

Derivation of kinetic equation for the inhibition of subtilisin by PMSF as monitored by *p*-nitrophenyl acetate. Let S = concentration of *p*-nitrophenyl acetate and P = concentration of *p*-nitrophenol. The conditions are: $S = S_0 =$ constant $\ll K_m^S$ and $I = I_0 =$ constant

[2] Neet and Koshland (1966) have made similar observations. Professor Koshland suggested such experiments.

[3] $k_{cat}/K_m = k_2/K_s$, where k_2 is the rate of acylation and K_s is the dissociation constant of the enzyme–substrate complex.

[4] The amino acid compositions of the Nagarse enzyme (Matsubara *et al.*, 1965) and of the Nova enzyme (Johansen and Ottesen, 1964) are the same within experimental error.

$\ll K_m^I$.

$$\left(\frac{dP}{dt}\right)_{\text{spontaneous}} = V_{SP} = k_{SP}S_0 \quad \text{(i)}$$

$$\left(\frac{dP}{dt}\right)_{\text{enzymatic}} = V_e = \frac{k_2}{K_s}S_0E \quad \text{(ii)}$$

Since the enzyme will be inactivated

$$\frac{-dE}{dt} = k_I I_0 E \quad \text{(iii)}$$

Integration of eq iii yields

$$E = E_0 e^{-k_I I_0 t} \quad \text{(iv)}$$

Substitution of eq iv into eq ii yields

$$V_e = \frac{k_2}{K_s}S_0E_0e^{-k_I I_0 t} \quad \text{(v)}$$

Since $V_{\text{exp}} = V_{\text{sp}} + V_e$, then

$$V_{\text{exp}} = \left(\frac{dP}{dt}\right)_{\text{exp}} = k_{SP}S_0 + \frac{k_2}{K_s}S_0E_0e^{-k_I I_0 t} \quad \text{(vi)}$$

Integration of eq vi gives

$$P = k_{SP}S_0t - \frac{k_2/K_sS_0E_0}{k_I I_0}(e^{-k_I I_0 t} - 1) \quad \text{(vii)}$$

Acknowledgment

The authors thank Professor Ferenc J. Kézdy for valuable discussions concerning the kinetics and Professor D. E. Koshland, Jr., for informing us of his parallel research.

References

Balls, A. K., and Jansen, E. F. (1952), *Advan. Enzymol. 13*, 321.
Bender, M. L., and Brubacher, L. J. (1964), *J. Am. Chem. Soc. 86*, 5333.
Bender, M. L., Kézdy, F. J., and Zerner, B. (1963), *J. Am. Chem. Soc. 85*, 3017.
Bender, M. L., Pollock, E. J., and Neveu, M. C. (1962), *J. Am. Chem. Soc. 84*, 595.
Bernhard, S. A., Hershberger, E., and Keizer, J. (1966), *Biochemistry 5*, 4120.
Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Brubacher, L. J., and Bender, M. L. (1966), *J. Am. Chem. Soc. 88*, 5871.
Gold, A. M., and Fahrney, D. (1964), *Biochemistry 3*, 783.
Johansen, G., and Ottesen, M. (1964), *Compt. Rend. Trav. Lab. Carlsberg 34*, 206.
Kézdy, F. J., and Bender, M. L. (1962), *Biochemistry 1*, 1097.
Lawson, W. B., and Schramm, H. J. (1965), *Biochemistry 4*, 377.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Matsubara, H., Kasper, C. B., Brown, D. M., and Smith, E. L. (1965), *J. Biol. Chem. 240*, 1125.
Neet, K. E., and Koshland, D. E., Jr. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1606.
Peller, L., and Alberty, R. A. (1959), *J. Am. Chem. Soc. 81*, 5907.
Photaki, I., and Bardakos, V. (1965), *J. Am. Chem. Soc. 87*, 3489.
Polgar, L. (1964), *Acta Physiol. Acad. Sci. Hung. 25*, 303.
Polgar, L., and Bender, M. L. (1966), *J. Am. Chem. Soc. 88*, 3153.
Schoellmann, G., and Shaw, E. (1963), *Biochemistry 2*, 252.
Whitaker, J. R. (1962), *J. Am. Chem. Soc. 84*, 1900.
Zioudrou, C., Wilchek, M., and Patchornik, A. (1965), *Biochemistry 4*, 1811.