Conformation of the Active Site of Thiolsubtilisin: Reaction with Specific Chloromethyl Ketones and Arylacryloylimidazoles†

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ABSTRACT: The conformation of the active site of thiolsubtilisin, prepared from subtilisin by transformation of the active site Ser to Cys, was compared with that of subtilisin by kinetic and spectroscopic methods. Carbobenzyloxy-L-alanylglycyl-L-phenylalanine chloromethyl ketone inhibited thiolsubtilisin $\sim 10^2$ times faster than subtilisin; alkylation occurred at the sulfhydryl rather than the imidazolyl group of the active site. pH dependence of the inhibition is different from that of the reaction between a simple thiol with haloacetamide. Furthermore, several native chromophoric arylacryloyl-thiolsubtilisins and arylacryloyl-subtilisins showed similar red shifts when compared with their denatured forms. The rate of deacylation of arylacryloyl-thiolsubtilisins was faster than (or of the same order of magnitude as) the deacylation rate of the analogous arylacryloyl-subtilisins in 30% dioxane (v/v), pH 5–10. The deacylation rate–pH profiles of these arylacryloyl-thiolsubtilisins in 30% dioxane all give pK values of 7.7 which is identical with the pK in the deacylation of acyl-subtilisins. These facts strongly suggest that the active-site conformation remains intact on conversion from subtilisin to thiolsubtilisin. The low esterase and peptidase activities of thiolsubtilisin are most likely due to the relatively low basicity of –SH (compared with –OH).

Thiolsubtilisin (SHSTL)[1] has been synthesized from subtilisin (STL) in which the hydroxyl group of the active site serine is transformed to a sulfydryl group. The loss of enzymatic activity toward specific substrates is remarkable (Polgar & Bender, 1967; Neet et al., 1968). Although some physicochemical properties of SHSTL and its reactions with *p*-nitrophenyl acetate and *trans*-cinnamoylimidazole were studied, either distortion of the active site conformation or a change in the basicity of the nucleophile could be responsible for the drastic reduction of enzyme activity after transformation.

In attempts to assess these possibilities, the present investigation compared SHSTL with STL in (1) the reactivities toward highly specific peptidyl chloromethyl ketones and (2) the spectra and the deacylation rates of several pairs of chromophoric arylacryloyl-enzymes. These are sensitive tests of the conformation of the active site, indicating whether or not it remains intact during the transformation from STL to SHSTL.

Experimental Section

Materials. STL was converted to SHSTL by using the procedure of Polgar & Bender (1967). After the Sephadex G-25 step, the SHSTL was further purified by using a 20 × 1.5 cm column of *p*-chloromercuribenzoate derivatized agarose (Affi-Gel 501, Bio-Rad Labs). Material which did not bind to the column was discarded, and SHSTL was eluted by addition of 0.25 M mercaptoethanol.

Active-site concentrations of SHSTL were determined by titration with *p*-nitrophenyl trimethylacetate under conditions of $[S]_0 \gg [E]_0$ and $[S]_0 \gg K_m$, $k_2 \gg k_3$ (Bender et al., 1966). STL concentrations were determined by use of second-order *p*-nitrophenyl acetate rate assays (Polgar & Bender, 1967). The number of sulfhydryl groups per molecule of SHSTL was determined by using DTNB (Ellman, 1959).

SHSTL was not inhibited by 10^{-4} M phenylmethanesulfonyl fluoride (Polgar & Bender, 1967) or by 10^{-2} M diisopropyl fluorophosphate. However, activity was totally abolished by inhibition with 5×10^{-3} M PCMB (Neet et al., 1968; Polgar & Bender, 1967).

ZAGPCK was a gift from Dr. K. Morihara (1974), and Tos-PheCH_2Cl was obtained from Sigma Chemical Co.

Buffers and organic solvents used were at least of reagent grade. Mallinckrodt Spectrograde dioxane and Nanograde acetonitrile were used as solvents for the substrates or inhibitors.

Kinetics. Spectrophotometric studies were carried out on a Cary 14 thermostated at 25 °C. pH values of the reaction mixtures were determined with a Radiometer Model 26 pH meter. The hydrolyses of *p*-nitrophenyl butyrate (Bender et al., 1963), Z-Gly-Gly-L-Tyr methyl ester, and Z-Gly-Gly-Trp methyl ester (Philipp & Bender, 1974) were measured at 400, 237, and 300 nm, respectively.

The kinetics of inhibition by irreversible inhibitors can be analyzed by using the scheme

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_3} E\text{–}I$$

where E·I represents a noncovalent complex of enzyme and inhibitor, E–I the covalent product, and k_3 the rate constant of inhibition. Pseudo-first-order kinetics were observed for the inhibition reactions, if [I] was sufficiently greater than [E]. The observed first-order inhibition rate constant, k_{obsd}, is given by eq 1 (Kitz & Wilson, 1962)

$$\frac{1}{k_{obsd}} = \frac{1}{k_3}\left(\frac{K_I}{[I]} + 1\right) \quad (1)$$

where $K_I = [E][I]/[E{\cdot}I]$. If the inhibitor concentration used is much smaller than K_I, eq 1 reduces to

$$\frac{k_{obsd}}{[I]} = \frac{k_3}{K_I} \quad (2)$$

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[1] Abbreviations used: SHSTL, thiolsubtilisin; STL, subtilisin; Im, imidazole; ZAGPCK, carbobenzyloxy-L-alanylglycyl-L-phenylalanine chloromethyl ketone; Tos-PheCH_2Cl, tosyl-L-phenylalanine chloromethyl ketone; Tos-LysCH_2Cl, tosyl-L-lysine chloromethyl ketone; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate; DMAC, 4-dimethylaminocinnamoyl; IA, *trans*-indoleacryloyl; Leu-PheNH_2, L-leucyl-L-phenylalaninamide; GlyNH_2, glycinamide; Ac, acetyl.

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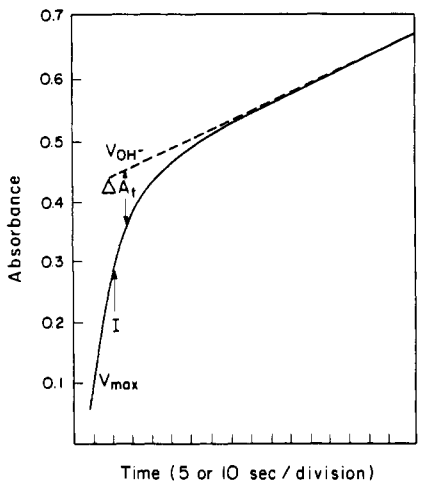

FIGURE 1: A schematic representation of the hydrolysis of *p*-nitrophenyl butyrate by SHSTL and the course of inhibition by ZAGPCK at neutral and higher pHs. The spontaneous hydrolysis rate (v_{OH}) leveled off at pH ≤7. Addition of ZAGPCK is denoted by an arrow.

Based on the data of Morihara (1974), the K_m's of Ac-(Ala)$_2$-Phe-OMe, Z-(Ala)$_2$-Lys-OMe, and Z-Ala-Gly-Leu-NH_2 are 0.32, 0.16, and 66.7 mM, respectively. Thus, the K_I of ZAGPCK may be assumed to be ≥0.1 mM and, thus, is much higher than the inhibitor concentration of 10^{-5} M used here. Accordingly, k_3/K_I can be determined by eq 2.

The rate of inactivation of STL Novo by ZAGPCK was followed by adding a two- to threefold excess of inhibitor (freshly prepared in dioxane) to STL. Aliquots of the mixture were withdrawn at various times and assayed with *p*-nitrophenyl butyrate under saturation conditions ($[S]_0 \gg K_m$). The zero-order rates of hydrolysis, $v = k_{cat}[E]$, were followed for about 100 s and corrected for the buffer-catalyzed rate from control runs. A control with the same concentration of dioxane (2.6 or 5.0% v/v) without inhibitor showed a negligible change of enzyme activity at the end of 1 h and gave the zero-order rate (v_0) at $t = 0$. The rate of inhibition of STL was studied at various concentrations of ZAGPCK, and $k_{obsd}/[I] = k_3/K_I$ was determined from log ($v_0 - v_t$) vs. time.

On the other hand, the inhibition of SHSTL by ZAGPCK was so fast that it could not be followed by the above method. Thus, the decrease in rate of activity was followed by adding ZAGPCK to a buffered solution of SHSTL immediately after *p*-nitrophenyl butyrate was introduced. Conditions were used in which $[S] \gg K_m$ and $[S] \gg [I] \gg [E]$ using an [E] large enough to observe the catalyzed reaction (over the spontaneous hydrolysis), but low enough so that the course of inhibition could be followed. The pseudo-first-order inhibition rate constant, k_{obsd}, was obtained from the decrease of rate of the enzymatic hydrolysis with time. $k_{obsd} = [\ln (\Delta A_{t_1}/\Delta A_{t_2})]/(t_2 - t_1)$, where ΔA_{t_1} and ΔA_{t_2} are absorbance differences related to the concentrations of active enzyme at t_1 and t_2 (see Figure 1). The inhibition of SHSTL by ZAGPCK followed good pseudo-first-order kinetics at various pH values.

The inhibition of STL and SHSTL with Tos-PheCH$_2$Cl was studied in a similar manner.

For amino acid analysis in a Durrum D500 analyzer, ZAGPCK-inhibited enzymes were hydrolyzed in constant boiling 6 N HCl (24 h at 110 °C).

Acylation of Enzymes and Deacylation of Arylacryloyl-Enzymes. The acylation of 1.0×10^{-4} M SHSTL or of STL (Novo) by 4.0×10^{-5} M DMAC-Im (Breaux & Bender, 1976) or IA-IM (Johansen et al., 1969) was carried out at pH 5.2, 0.04 M acetate buffer; the reactions went to completion in 10–20 min. Then aliquots of acyl-enzyme and excess enzyme, with or without gel filtration, were diluted with various buffers. The spectra were taken vs. the same concentration of enzyme.

The difference spectra of denaturated acyl-enzymes vs. denatured enzymes were obtained similarly by adding the calculated amount of 2 M HCl and 10% NaDodSO$_4$ to the native enzymes and acyl-enzymes leading to final conditions of pH 3.5–3.7 and 1.0% NaDodSO$_4$.

The first-order deacylation rates (k_3) at various pHs were studied at the λ_{max} in the presence and absence of dioxane. The following buffers were used: pH 2.0–3.0 citrate; pH 3.7–4.0 formate; pH 4.0–5.0 acetate; pH 6.0–7.9 phosphate; pH 8.0–9.0 Veronal; pH 9.0–10.7 carbonate. In 30% dioxane, the pH ranges of the buffers were approximately 0.7 unit higher. The spectra of the products of deacylation were checked after completion of the reaction.

The second-order rate constant (k_n) for the deacylation by an added nucleophile N was calculated by dividing ($k_{obsd} - k_3$) by [N]. Here k_3 (the first-order rate constant for deacylation in buffer in the absence of added nucleophile) was assumed not to be changed by the presence of $\leq 10^{-2}$ M nucleophile. In these experiments, various nitrogen nucleophiles having different pK_a values were compared. The reactions were run at pH 8.95, where the nucleophiles are primarily in their unprotonated form and the deacylation rate is on a plateau of the pH profile.

Table I: Rates of Inhibition of Proteases by Specific Chloromethyl Ketones

enzymes	inhibitors	pH	k_3/K_I (M^{-1} s^{-1})
STL	ZAGPCK	7.9	48,[a] 35[b]
	ZAGPCK	7.0	18–37[c]
	ZPCK	7.0	≤0.1[c]
SHSTL	ZAGPCK	7.8	1.8×10^3[a]
	ZAGPCK	6.7	8.4×10^2[a]
	ZAGPCK	5.1	1.5×10^2[a]
	Tos-PheCH$_2$Cl	7.8	$\leq 10^{-4}$
papain	Tos-LysCH$_2$Cl	5.2	3×10^3[d]
	Tos-PheCH$_2$Cl	9.5	1.1×10^3[e]

[a] 2.6% (v/v) dioxane. [b] 5.0% (v/v) dioxane. [c] From Morihara et al. (Morihara et al., 1971; Morihara & Oka, 1970; Morihara, 1974). [d] From Whitaker et al. (1968). [e] From Brubacher & Bender (1967).

Results

Enzymatic Activity of SHSTL toward Good Substrates of STL. SHSTL, purified by affinity chromatography on an organomercurial agarose column, did not catalyze the hydrolysis of Z-Gly-Gly-Tyr methyl ester or Z-Gly-Gly-Trp methyl ester, which are the best substrates for STL (Philipp & Bender, 1974). Acyl-enzymes did not accumulate in similar cases (Neet et al., 1968). The results indicate a reduction in acylation rate of about 10^5–10^6-fold with respect to STL. However, both STL and SHSTL hydrolyzed *p*-nitrophenyl alkanoates (Philipp et al., 1979).

Inhibition by Specific Chloromethyl Ketones. ZAGPCK inhibited both STL and SHSTL at a rate 10^5–10^6 greater than that in the case of nonenzymic model reactions. In Table I, the second-order inhibition constants (k_3/K_I), determined as described in the Experimental Section, are listed together with the values for papain.

The results in Table I show that the rate of ZAGPCK inhibition of SHSTL is about 10–10^2 times faster than that of STL and is of the same order of magnitude as that of the Tos-PheCH$_2$Cl inhibition of papain.

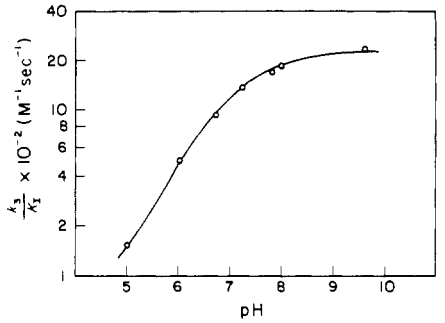

FIGURE 2: The pH profile of the second-order inhibition rate constants of SHSTL by ZAGPCK at 25 °C. The inhibition rates at pH 8.0 and 9.65 were calculated from the relative rates at 2 °C by assuming the same pH dependence at both temperatures.

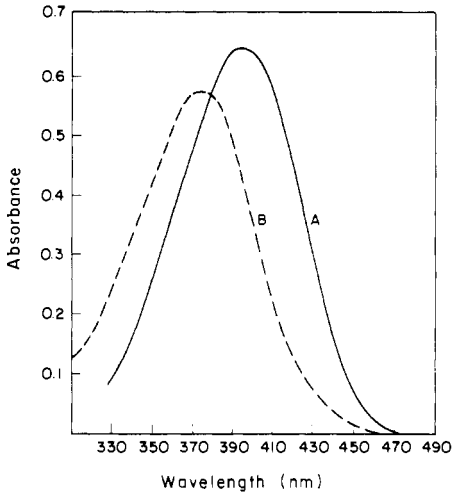

FIGURE 3: Difference spectra of DMAC-STL. (A) (—) Acyl-enzyme vs. enzyme, λ_{max} = 398 nm; (B) (---) denatured acyl-enzyme vs. denatured enzyme, λ_{max} = 372 nm.

Furthermore, titration of SHSTL (after completion of inhibition by ZAGPCK) with DTNB showed a considerable decrease of the titratable sulfhydryl group. When the amino acid compositions of SHSTL before and after ZAGPCK inhibition were compared, the number of histidine residues was not reduced. Thus, the alkylation site in SHSTL was found to be the cysteine *sulfhydryl* group as in the case of papain, in contrast to alkylation of the *imidazolyl* group in the ZAGPCK inhibition of STL (Morihara et al., 1971). Considering that a sulfhydryl group is generally $10–10^2$ more reactive than an imidazolyl group in model alkylation reactions (Shaw, 1975; Whitaker & Perez-Villasenor, 1968), the binding specificities of SHSTL and STL must be very similar to each other.

Figure 2 shows the pH profile of the inhibition rate of SHSTL by ZAGPCK. It is remarkably different from that of the reaction between a simple thiol compound with haloacetamide (Polgar et al., 1973).

In contrast to the fast inhibition of STL and SHSTL by ZAGPCK, Tos-PheCH$_2$Cl showed only a slow inhibition of STL (Morihara, 1974; Shaw, 1975) and SHSTL. When 4.0 × 10^{-4} M Tos-PheCH$_2$Cl was incubated with 2.5 × 10^{-5} M SHSTL in pH 8.65, 0.15 M Veronal buffer, 10% dioxane, for 24 h at 25 °C, only 5% inhibition with respect to the control was observed. This suggests that the k_3/K_I value is less than 1 × 10^{-4} M^{-1} s^{-1} for Tos-PheCH$_2$Cl toward SHSTL (Table I).

Spectra of DMAC- and IA-Enzymes. The difference spectra of DMAC-STL, DMAC-SHSTL, and their denatured forms are shown in Figures 3 and 4. DMAC-STL has a λ_{max} of 398 nm, whereas DMAC-SHSTL has a λ_{max} of 427 nm. On denaturation, both λ_{max}'s shifted to values which are almost identical with those of DMAC derivatives of *N*-Ac-Ser-NH$_2$ and *N*-Ac-Cys-NH$_2$, respectively. Similar results were obtained for the IA-enzymes.

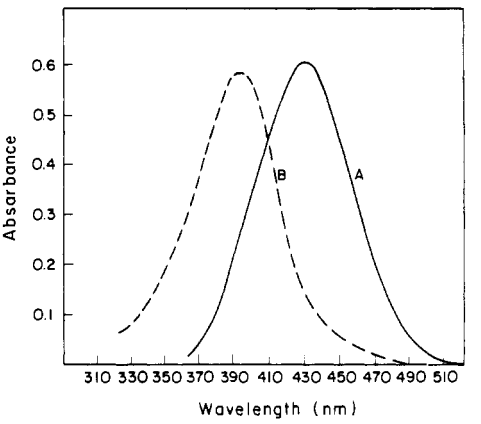

FIGURE 4: Difference spectra of DMAC-SHSTL. (A) (—) Acyl-enzyme vs. enzyme, λ_{max} = 427 nm; (B) (---) denatured acyl-enzyme vs. denatured enzyme, λ_{max} = 392 nm.

Table II: Absorption Maxima[a] of Arylacryloyl-enzymes and Models

acyl-enzymes[b] of	IA-E native	IA-E denatured	DMAC-E native	DMAC-E denatured	*trans*-cinnamoyl-E[c] native	*trans*-cinnamoyl-E[c] denatured
STL Novo	349	339	398	372	289	284
SHSTL Novo	377	365	427	392	311	306
papain	398	373			326	306
N-Ac-Cys-NH$_2$	363		390		306	
N-Ac-Ser-NH$_2$	333		370		281	

[a] Absorption maxima are in nanometers. [b] The acyl derivatives at the bottom are models. [c] References in text.

The λ_{max} of the arylacryloyl-enzymes including those for the *trans*-cinnamoyl-enzymes (Polgar & Bender, 1967; Brubacher & Bender, 1966) and acyl-papains (Hinkle & Kirsch, 1970; Bernhard et al., 1965) as well as those of models for purposes of comparison are summarized in Table II.

The λ_{max}'s of thiol esters were more red-shifted than oxygen esters by 22–29 nm in the denatured arylacryloyl-enzymes or the nonenzymatic models, possibly because of 3d orbital overlap of the sulfur atom of the thiol ester. More importantly, the λ_{max}'s of the native acyl-SHSTL are greater than those of the homologous native acyl-STL by 22–29 nm, and the shift of λ_{max} of each acyl-SHSTL on denaturation is comparable in magntiude to that of the homologous acyl-STL on denaturation (see Table II). This suggests that the structure of an acyl-SHSTL is very similar to that of an acyl-STL.

Deacylation of DMAC- and IA-Enzymes. The deacylation rate of DMAC-STL increases with pH and depends on a group of pK = 7.7; this pH dependency is very similar to that for IA-STL (Johansen et al., 1969). The k_3 (lim) values are 2.5 × 10^{-2} and 1.9 × 10^{-2} s^{-1} for DMAC-STL and IA-STL, respectively. The addition of 30% dioxane to the buffers showed no effect on the deacylation of DMAC- and IA-STL.

Interestingly, however, dioxane was found to greatly accelerate the deacylation of both IA- and DMAC-SHSTL. As shown in Table III, the increases of rate by 30% dioxane are up to 10^2, taking into account that the apparent pH of the buffer solution is raised by 0.6–0.8 pH unit in the presence of 30% dioxane. The higher the concentration of dioxane up

Table III: Dependence of Deacylation Rate (k_3) of DMAC-SHSTL and IA-SHSTL on Dioxane Concentration

% dioxane (v/v)	pH[a]	$k_3 \times 10^4$ (s^{-1}) DMAC-SHSTL	$k_3 \times 10^4$ (s^{-1}) IA-SHSTL
0	8.65	14.9	1.58
10	8.88	189	98.1
20	9.13	277	133
30	9.38	426	216
40	9.31	479	242

[a] pH reading after the addition of dioxane at the concentration indicated, in borate buffer 0.10 M at 25.0 °C.

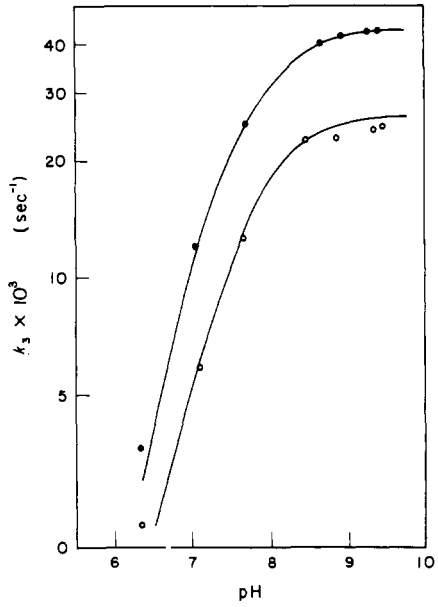

FIGURE 5: Dependencies of the deacylation rate (k_3) on pH, in buffers with 30% dioxane (v/v), 25 °C. (O—O) For IA-SHSTL; (●—●) for DMAC-SHSTL. The solid lines represent theoretical curves for pK_a = 7.7.

to 40 or 50%, the faster was the deacylation rate constant (k_3). The k_3 (lim) of the hydrolysis of DMAC- and IA-SHSTL in 30% dioxane were determined to be 4.3×10^{-2} and 2.6×10^{-2} s^{-1}, respectively, and the pK_a values were 7.7 (Figure 5), which are similar to those for the hydrolysis of DMAC-STL in 30% dioxane.

Acceleration by dioxane was observed only for the deacylation of native acyl-SHSTL, and dioxane showed no acceleration of denatured DMAC-SHSTL.

Several other organic solvents, including tetrahydrofuran, dimethyl sulfoxide, dimethoxyethane, and dimethylformamide, did not change the deacylation rate as dioxane did. DMAC- and IA-SHSTL were denatured in the presence of ≥10% tetrahydrofuran.

Dioxane showed little effect on the acylation of SHSTL reactions. When 20 or 30% (v/v) dioxane was added to a buffered solution containing *p*-nitrophenyl butyrate and SHSTL, k_{cat} and K_m^{-1} decreased slightly. No hydrolysis of specific substrates (e.g., Z-Gly-Gly-Trp methyl ester) by SHSTL could be detected even in the presence of dioxane. Earlier experiments also failed to activate SHSTL by various conformation modulators (Neet et al., 1968).

The partitioning of DMAC-SHSTL between water and nitrogen nucleophiles, which also indicates a thiol ester intermediate in SHSTL reactions (Polgar & Bender, 1967), was examined by use of $GlyNH_2$ (nonspecific) and Leu-$PheNH_2$ (specific) as nucleophiles. The rate constants of the deacylation by these nucleophiles (k_n) (Experimental Section) were 7.8 $\times 10^{-2}$ M^{-1} s^{-1} with $GlyNH_2$ and 3.2 M^{-1} s^{-1} with Leu-$PheNH_2$, respectively.

Discussion

The spectroscopic and kinetic studies in the present investigation show the following. (1) The inhibition of SHSTL by ZAGPCK is about 10^2 faster than its inhibition of STL, which is definitely attributable to the difference of the alkylation sites (SH and Im, respectively). Furthermore, both SHSTL and STL are resistant to Tos-$PheCH_2Cl$ inhibition. This similarity in inhibitor specificity is a strong indication that the $S_1...S_4$ binding sites remain intact during the oxygen to sulfur transformation since the specificities of the chloromethyl ketone derivates of specific amino acids and of peptides with respect to $S_1...S_4$ sites are as strict as are those of the substrates (Powers, 1977; Shaw, 1975). (2) The shift of the λ_{max} of chromophoric arylacryloyl-SHSTL on denaturation is comparable in magnitude to that of the homologous arylacryloyl-STL on denaturation. These facts strongly indicate that the active site conformation remains intact on conversion of STL to SHSTL. (3) The comparable deacylation rates of acyl-SHSTL and acyl-STL in 30% dioxane and their similar pH dependences are consistent with an intact active site conformation, although this is not conclusive evidence. It should be pointed out that the rates of alkaline hydrolysis of thiol esters are of the same of magnitude as those of oxygen esters (Rylander & Tarbell, 1950; Morse & Tarbell, 1952).

The large acceleration of deacylation by $GlyNH_2$ and Leu-$PheNH_2$ of arylacryloyl-SHSTL, which has been also observed with papain, has been attributed to S_1,S_2' and P_1,P_2' interactions, supports an intact active site conformation of SHSTL. The greater effectiveness of Leu-$PheNH_2$ over $GlyNH_2$, in spite of their similar p*K*s, suggests that specific interactions between these nucleophiles and the S_1,S_2' subsites (on the C terminal side of the scissile peptide bond) might occur. These specific interactions favoring Leu-$PheNH_2$ over $GlyNH_2$ also operate in STL (Morihara, 1974).

No conformational distortion in SHSTL with respect to STL was indicated by the parallel specificity of SHSTL and STL in the hydrolysis of straight chain *p*-nitrophenyl alkanoates (Philipp et al., 1979).

Consequently, lack of a high hydrolytic activity of SHSTL toward specific substrates, and low rate of acylation, should be attributed simply to conversion of the nucleophilic site from OH to SH, and the smaller basicity of RSH (or RS^-) than ROH (or RO^-). According to the "Hard and Soft Acids and Bases" rule of Pearson et al. (Edwards & Pearson, 1962; Pearson & Songstad, 1967), and nonenzymatic experiments (Bunnett, 1963; Edwards & Pearson, 1962), RO^- and ROH are better nucleophiles than RS^- or RSH toward the carbonyl carbon atom of esters or amides. Thus, nonenzymatic experiments and arguments bear out our conclusions.

It should not be too surprising that none of the well-defined serine proteases including STL contains a hydrogen bond between the reactive serine and histidine residues of the putative charge-relay system as demonstrated by the X-ray crystallographic data (Matthews et al., 1977). Recent molecular orbital calculations suggest slight activation of the reactive serine in native serine proteases, i.e., the serine is not intrinsically a better nucleophile than ordinary ROH, but in a good position to attack the carbonyl carbon atom of the substrate (Kraut, 1977). It cannot be concluded whether there is a hydrogen bond between Cys-221 and His-64 in SHSTL, although the pH profile at ZAGPCK inhibition of SHSTL shows an enhanced rate at low and neutral pH. The data

herein suggest that the relatively low basicity of Cys-221 in SHSTL toward substrates cannot be compensated or corrected by the presence of an intact His···Asp couple at the active site.

The reactivity of SHSTL is much poorer than a native thiol protease such as papain. To compensate for the low basicity of the nucleophilic RS^- at the active site, the acylation step in natural thiol proteases probably involves protonation of the leaving group before or during the transition state or tetrahedral intermediate is formed. In contrast, the protonation of the leaving group must occur later than bond formation between the serine oxygen atom and the carbonyl carbon atom of the substrate in serine proteases (Bizzozero & Zweifel, 1975; Fastrez & Fersht, 1973). In support of these mechanistic arguments, the active site geometries of natural thiol and serine proteases were found to be only slightly different. Although both groups of proteases use similar features such as the His-Asp proton transfer system and the oxyanion hole, the spatial relationships between these catalytic residues are not the same (Garavito et al., 1977). This is reflected in differences in the extent of the red-shift of the absorption spectra reported here, in the resonance Raman spectra between acyl-papains and acyl-chymotrypsins (Carey et al., 1978), and in the pH profiles of the inhibition rates of SHSTL and papain by iodoacetamide (Polgar et al., 1973).

The effect of dioxane in increasing the deacylation rate of arylacryloyl-SHSTL is not simply a dielectric effect because urea, tetrahydrofuran, dimethyl sulfoxide, dimethylformamide, dimethoxyethane, and ethanol had no such effect on deacylation. This might be associated with acceleration of the deacylation rate of arylacryloyl-papains by dioxane and several other organic solvents (Hinkle & Kirsch, 1970).

The deacylation rate of denatured acyl-SHSTL was not accelerated by dioxane. Therefore, the dioxane effect observed with the native acyl-enzyme is most likely a specific conformational modulation at the intact active site. The effect can be correlated with the unusual configuration of arylacryloyl-SHSTL, which is very specific in terms of the structure of the acyl group and its relation to the active site side chains of the enzyme.

In conclusion, the present study indicates that the conformation of the active site remains intact in the transformation of STL to SHSTL. The inactivity of SHSTL in contrast to STL can be attributed to the lower basicity or nucleophilicity of Cys-SH than that of Ser-OH at the active site. This conclusion is based on spectroscopic and kinetic studies of various acyl-enzymes and the inhibition of both STL and SHSTL by specific chloromethyl ketones. The evidence confirms preliminary X-ray crystallographic studies (Alden et al., 1970) and earlier physicochemical studies on SHSTL (Neet et al., 1968). This conclusion is important for any attempt to clarify the mechanism of proteases by comparing the enzymatic activity of STL, SHSTL, and native thiol proteases.

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