Comparison of the Kinetic Specificity of Subtilisin and Thiolsubtilisin toward *n*-Alkyl *p*-Nitrophenyl Esters†

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ABSTRACT: The *p*-nitrophenyl esters of straight-chain fatty acids were used as substrates of the enzyme subtilisin Novo (EC 3.4.4.16) and its chemically produced artificial enzyme thiolsubtilisin. Subtilisin and thiolsubtilisin pH–activity profiles were determined, and kinetic effects of the active site O–S substitution were observed. Among the substrates tested, both enzymes show highest specificity with *p*-nitrophenyl butyrate. It was also found that subtilisin is more sensitive to changes in substrate chain length than is thiolsubtilisin. Second-order acylation rate constants (k_2/K_s) are remarkably similar for both enzymes. However, thiolsubtilisin deacylation rate constants and K_m values are lower than analogous subtilisin constants. While thiolsubtilisin deacylation rate constants give a pH profile identical with that of subtilisin, the pH profile of thiolsubtilisin acylation rate constants shows an active site pK value lowered from the subtilisin pK of 7.15 and exhibits an inflection point at pH 8.45, which is absent in subtilisin.

Thiolsubtilisin (SHSTL[1]) is an enzyme formed by an OH to SH substitution on the active site serine-221 side chain of subtilisin (STL) (Polgar & Bender, 1966; Neet & Koshland, 1966). Previous studies have shown that this substitution destroys protease activity (Neet et al., 1968) and greatly decreases deacylation rates of all other substrates (Neet et al., 1968; Polgar & Bender, 1967). The substitution, however, has been shown to have only a small effect on k_2/K_s (eq 1)

$$E + S \overset{K_S}{\rightleftharpoons} ES \overset{k_2}{\longrightarrow} EP_2 \overset{k_3}{\longrightarrow} E + P_2 \qquad (1)$$
$$\searrow P_1$$

for a nonspecific STL substrate, NP acetate (Polgar & Bender, 1967). However, O–S substitution has a considerable effect on the acetyl-enzyme deacylation rate constants (k_3 in eq 1) and in NP acetate K_m values.

In contrast to the situation with NP acetate, acylation has been shown to be the most hindered step in hydrolysis of specific ethyl esters and amides by SHSTL (Neet et al., 1968). Deacylation of specific and nonspecific acyl-SHSTLs is observable and is 10–100-fold slower than deacylation of analogous acyl-STLs (Neet et al., 1968). Yet, the acylation of SHSTL by nonactivated amides and ethyl esters is totally unobservable (Neet et al., 1968; Tsai, 1977; Tsai & Bender, 1979).

Attempts to explain the existing kinetic data have been made problematical since SHSTL has been studied by using only two nonspecific substrates, NP acetate (for k_2/K_s and k_3) and *N-trans*-cinnamoylimidazole (for k_3). In addition, a value for k_2/K_s at a single pH has been obtained for ethyl thiolacetate (Polgar, 1972).

Study of more specific substrates has been hindered both by the unreactivity of nonactivated esters and amides and by the difficulty of obtaining SHSTL preparations which do not catalyze the hydrolysis of specific NP esters in the presence of thiol group inhibitors. Even highly purified SHSTL preparations show this behavior (Polgar & Bender, 1969).

In order to obtain more specific SHSTL substrates which nonetheless exhibit only SH-dependent catalysis, a series of NP alkanoates was considered. A previous study of STL Carlsberg has shown that alkyl chain length increases in such substrates result in larger values of k_2/K_s (Graae, 1954).

These substrates also have two other advantages. They are less activated than more specific acylamino acid ester (Silver et al., 1970) and exhibit lower spontaneous hydrolysis rates; they also have the advantage that specificity can be correlated with small, incremental changes in substrate structure, in a way not possible for most acylamino acid substrates.

Materials and Methods

Subtilisin (STL) Novo was purchased as Bacterial Proteinase Novo from the Enzyme Development Corp. Thiolsubtilisin (SHSTL) was prepared as before (Tsai & Bender, 1979).

NP acetate was obtained from Aldrich and recrystallized three times from absolute ethanol, mp 79 °C. NP butyrate, valerate, and caproate were purchased from Sigma and used without further purification. NP caprylate and NP laurate were purchased from Mann Laboratories and also used without further purification. PCMB immobilized on agarose was purchased from Bio-Rad.

NP propionate was prepared by refluxing a 1.5-fold excess of propionic anhydride together with sodium *p*-nitrophenolate for 1 h in ethyl acetate. The product was recrystallized four times from water, mp 62–63 °C (literature value 62–63 °C; Higgins & Lapides, 1947).

Buffers were prepared by using tables given in the *Biochemists' Handbook* (Long, 1961). All had an ionic strength of 0.1 M and utilized sodium acetate (pH <5.5), sodium phosphate, (pH 5.5–8), Tris-HCl and Veronal (pH 9), or sodium bicarbonate (pH <9).

Single pH kinetic determinations using SHSTL were done at pH 7.78 in sodium phosphate buffer with an ionic strength

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[1] Abbreviations used: Z, benzyloxycarbonyl; STL, subtilisin Novo; SHSTL, thiolsubtilisin; PCMB, *p*-chloromercuribenzoate; CHT, α-chymotrypsin; NP, *p*-nitrophenyl; μ, ionic strength.

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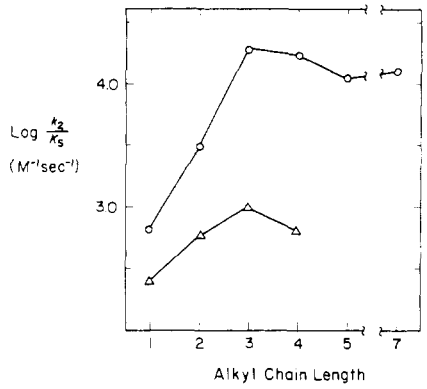


FIGURE 1: Dependence of k_2/K_s at optimal pH vs. alkyl group chain length in NP *n*-alkyl esters substrates catalyzed by STL (○) and SHSTL (△). Reaction conditions are given under Materials and Methods.

of 0.1 M (Long, 1961). pH 7.78 is the point of nearly maximum SHSTL k_2/K_s with NP butyrate and is a point where k_{cat} = 0.86 k_{cat} (lim).

STL pH independent kinetics were done at pH 9.2 in sodium bicarbonate buffer (μ = 0.1M) (Long, 1961). At this pH, k_2/K_s and k_{cat} essentially equal their limiting values.

Sigmoid pH dependencies were calculated in the usual manner by assuming single proton ionizations. The pH profile in Figure 5 was calculated by using eq 2. The theoretical

$$\frac{k_2}{K_s} = \frac{k_2/K_s(\text{lim})_1}{1 + \frac{(H)}{K_1} + \frac{K_2}{(H)}} + \frac{k_2/K_s(\text{lim})_2}{1 + \frac{(H)}{K_2}} \qquad (2)$$

curve was matched to the data points by iteration. This relationship was chosen by assuming that (1) pK_1 results from ionization of a single ionization group with the alkaline form of this group essential for enzymatic activity and that (2) pK_2 results from a single ionizable group with a substantial amount of enzymatic activity remaining on either side of the transition.

Results

k_2/K_s: Chain Length Profiles. Figure 1 shows acylation (k_2/K_s) rate constants for STL and SHSTL-catalyzed hydrolyses of *n*-alkyl-NP esters of varying alkyl chain length. It is apparent that a maximum exists with NP butyrate and that higher chain lengths do not produce a large reduction from the maximum rate. However, chain lengths longer than those presented in Figure 1 do cause decreases in rate, since NP laurate, under similar reaction conditions, has a STL k_2/K_s value of 865 M^{-1} s^{-1} (Philipp, 1971).

The maximum k_2/K_s value in Figure 1, 2 × 10^2 M^{-1} s^{-1}, may be compared with other STL values for more specific NP esters. Such esters are NP hippurate, k_2/K_s = 1.0 × 10^5 M^{-1} s^{-1} (at pH 7.7, μ = 0.1 M), NP *N*-Z-glycinate, k_2/K_s = 3.3 × 10^5 M^{-1} s^{-1}, and NP *N*-Z-L-phenylalaninate, k_2/K_s = 7.3 × 10^6 M^{-1} s^{-1} (pH 9.0, μ = 0.1 M; Philipp, 1971). It is apparent that, in terms of k_2/K_s, a continuous STL specificity range exists between poor substrates (such as NP acetate) and the good substrates (Morihara & Oka, 1973; Philipp, 1971; Philipp & Bender, 1974), such as NP *N*-Z-L-phenylalaninate.

Figure 3 shows that the specificity profiles of STL and SHSTL are quite similar, with the exception of a generally lower SHSTL reactivity. This strongly indicates that, in their productive complexes, these substrates bind to STL and SHSTL in a very similar manner. Only productive complexes affect the value of k_2/K_s (Kezdy & Bender, 1965). Since STL preferentially hydrolyzes derivatives of leucine (Morihara & Oka, 1973), it may be that these alkanoates fit in the enzyme site specific for the *n*-alkyl side chain of leucine.

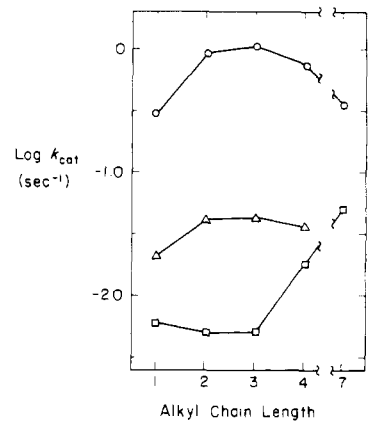


FIGURE 2: Dependence of log k_{cat} vs. alkyl group chain length in NP ester substrates, catalyzed by STL (○), SHSTL (△), and CHT (□). Values for CHT are averaged literature values; for n = 1, values from Kezdy & Bender (1962), Bender et al. (1964b), Marshall & Akgun (1971), and Cane & Wetlaufer (1966). For n = 2 and n = 3, values from Marshall & Akgun (1971), Cane & Wetlaufer (1966), and Fife & Milstein (1967). For n = 4, values from Marshall & Akgun (1971), Fife & Milstein (1967), and Enriquez & Gerig (1969).

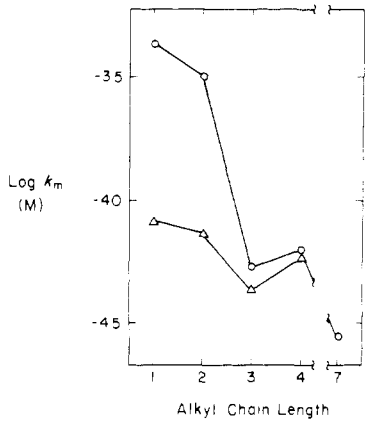


FIGURE 3: Dependence of log K_m vs. alkyl group chain length in NP *n*-alkyl ester substrates in reactions catalyzed by STL (○) and SHSTL (△). Reaction conditions are given under Materials and Methods.

k_{cat} Chain Length Profiles. k_{cat} chain length dependencies for STL and SHSTL are shown in Figure 2. The profiles for the two enzymes are nearly parallel, although the magnitude of the SHSTL constants is substantially lower than those of STL. The corresponding constants of CHT are even lower than those of SHSTL, since k_{cat} = k_3 in CHT reactions. Deacylation of the smallest possible acyl-enzymes, containing acyl groups that cannot possibly interact with amino acid side chain specificity sites, is much slower with CHT than with STL or SHSTL.

If it is assumed that SHSTL k_3 values are lower than those of STL values due to sulfur induced active site change, then nonspecific acyl-CHTs should have even more significant active site change (Figure 2) from the most productive configuration. This suggests, that, in the absence of specific substrate side chains, either the conformation of active site groups is less than optimal or the configuration of the acyl-intermediate is less than optimal in CHT. Such a possibility has been noted earlier (Cane & Wetlaufer, 1966), since indole, a specific substrate side chain group, accelerates deacylation of acetyl- and propionyl-CHT.

Serine proteases are known to hydrolyze thiolethyl esters more rapidly than ethyl esters (Hirohara et al., 1977). This

Table I: Catalytic Constants for the Hydrolysis of NP Trimethylacetate[a]

enzyme	k_{cat} (s^{-1})	K_m (M)	k_2/K_s (M^{-1} s^{-1})
subtilisin	4.7×10^{-2} (21)[b]	1.3×10^{-4} (2.5)[b]	370 (8)[b]
thiolsubtilisin	4.5×10^{-3} (9)[b]	1.5×10^{-4} (0.5)[b]	30 (19)[b]
chymotrypsin	1.3×10^{-4} (39)[b]	5.6×10^{-7} (2.0)[b]	232 (10)[b]

[a] Subtilisin and thiolsubtilisin kinetic constants were determined in pH 7.78 phosphate buffer (μ = 0.1 M) at 25 °C. Chymotrypsin values are from the literature (Bender & Hamilton, 1962) and were done in pH 8.2 Tris-HCl buffer (μ = 0.6 M) at 25.6 °C. [b] Numbers in parentheses are ratios of analogous NP propionate constants to NP trimethylacetate constants; NP propionate data are taken from Figure 3.

effect is due in part to k_2, the first-order acylation rate constant in eq 1 (Hirohara et al., 1977). Since deacylation can be considered to be the reverse of acylation by alkyl esters (Kezdy & Bender, 1965), it is apparent that electronic effects resulting from sulfur substitution should not hinder k_3. The k_3's nonetheless are tenfold lower for SHSTL than STL; this effect is probably due to differences in oxygen and sulfur covalent radii (Pauling, 1960).

In the cases of arylacryloyl-SHSTLs, k_3 values are more than 20-fold lower than those of the homologous arylacryloyl-STLs. However, addition of dioxane raises the SHSTL k_3 to a value very similar to that of STL (Tsai & Bender, 1979).

Since electronic effects and major active site distortions do not seem to be the cause of the lowered SHSTL k_3 values, the effect is probably due to differences in oxygen and sulfur covalent radii (see also Tsai & Bender, 1979).

Effect of Acyl Group Steric Hindrance on Kinetic Constants. Table I shows kinetic constants for hydrolysis of the hindered substrate, NP trimethylacetate, by STL, SHSTL, and CHT. Comparison of rate constants for this substrate shows that steric hindrance immediately adjacent to the active site affects STL and CHT deacylation rate constants somewhat more than it does the SHSTL rate constant. Since propionyl-SHSTL has a k_3 of 4.2×10^{-2} s^{-1}, propionyl-STL a k_{cat} of 0.99 s^{-1}, and propionyl-CHT a k_3 of 5×10^{-3} s^{-1} (Marshall & Akgun, 1971; Cane & Wetlaufer, 1966; Fife & Milstein, 1967), addition of sterically important methyl groups reduces k_{cat} by 9-fold in SHSTL, 31-fold in CHT, and 21-fold in STL.

These values suggest that the larger covalent radius of sulfur in SHSTL moves the substrate acyl group in the acyl-enzyme to an extent sufficient to make k_3 less susceptible to further steric hindrance.

K_m Chain Length Profile. The dependence of STL and SHSTL K_m values on substrate alkyl chain length is shown in Figure 4. It can be seen that the K_m values for the two enzymes converge at high chain lengths, while the general profile is similar, with a local minimum at n = 3. Interpretation of K_m effects is complicated by the fact that K_m is a complex constant, depending on both K_s and the ratio $k_3/(k_2 + k_3)$ (Gutfreund, 1955; Kezdy & Bender, 1965; Zerner & Bender, 1964) (eq 3).

The magnitude of K_m for these compounds (lower than that of other STL substrates) (Morihara & Oka, 1973; Ottesen & Svendsen, 1970) indicates that K_m is depressed by a high k_2/k_3 ratio in eq 3 (Zerner & Bender, 1964).

$$K_m = K_s k_3/(k_2 + k_3) \quad (3)$$

If K_s values for SHSTL are similar to K_s values for STL, an assumption supported by binding data for *N*-acetyl-D-tyrosinate (Neet et al., 1968), then the similarity of STL and

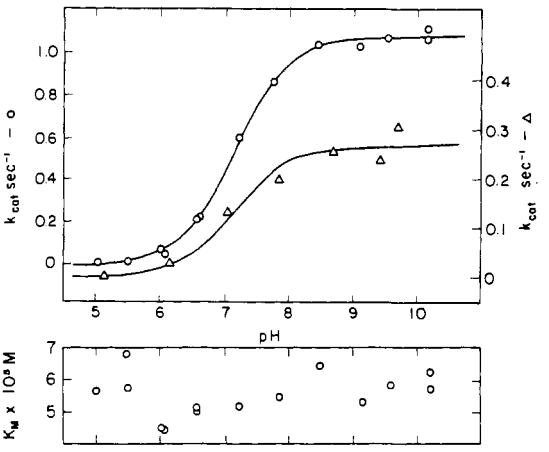


FIGURE 4: (Top) pH dependence of k_{cat} for STL (O) and SHSTL (Δ) and (bottom) pH dependence of K_m for STL (O), toward NP butyrate. The theoretical curves correspond to pK values for k_{cat} of 7.18 of STL and 7.15 for SHSTL. The curves correspond to values of k_{cat} (Lim) of 1.07 s^{-1} (STL) and 0.27 s^{-1} (SHSTL). μ = 0.1 M, 25 °C.

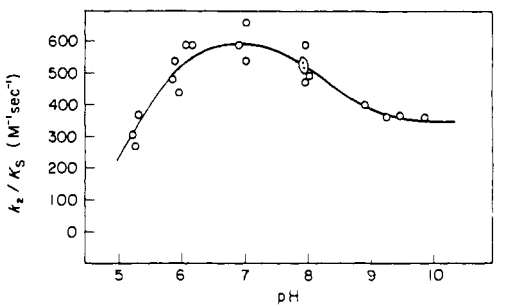


FIGURE 5: pH profile of k_2/K_s for the SHSTL-catalyzed hydrolysis of NP butyrate. The theoretical curve is discussed under Materials and Methods and corresponds to pK_1 = 6.08 and pK_2 = 8.45. k_2/K_s(lim) = 1550 M^{-1} s^{-1} and k_2/K_s(lim) = 1260 M^{-1} s^{-1}. μ = 0.1 M, 25 °C.

SHSTL K_m values for long chain esters implies similar k_2/k_3 ratios. Conversely, the low SHSTL K_m values for short chain esters imply higher SHSTL k_2/k_3 ratios for short chain esters.

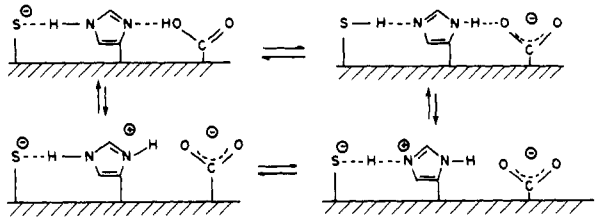
Since k_{cat} (or k_3) chain length profiles for STL and SHSTL are parallel (Figure 2), the effects of O–S transformation on k_3 are independent of chain length. It is apparent that k_2 values are diminished by O–S transformation primarily in long chain length substrates.

pH-Dependent Kinetic Constants. STL K_m values for neutral substrates have been shown to be pH independent between pH 4 and 10 (Polgar & Bender, 1967; Philipp, 1971; Glazer, 1967). This is confirmed in Figure 4, which shows the pH profile of k_{cat} and K_m for NP butyrate. The pK of k_{cat} (and k_2/K_s), 7.15, is the same for most STL substrates investigated in this laboratory (Polgar & Bender, 1967), including the most specific STL substrate known, *N*-Z-glycyl-glycyl-L-tyrosine methyl ester (Philipp & Bender, 1974).

Figure 4 shows that this pK of the acyl-enzyme is completely unaffected on O–S transformation. This figure shows the pH dependence of k_{cat} (presumably k_3) of *n*-butyryl-SHSTL. Since this pK in free native STL, acyl-STLs, and acyl-SHSTLs is identical, it is apparent that the pK of the active center is unaffected by acylation or acylthiolation.

Figure 5 shows the pH profile for acylation (k_2/K_s) of SHSTL by NP butyrate. The pH profile, in constrast to that of the native enzyme, is quite complex; it shows two pH-independent regions and two pK values. Most striking is the absence of the pK of 7.15 and its substitution by a pK of 6.08.

Scheme I



A lowered pK has been observed previously and has been discussed by Polgar & Bender (1969) in terms of cysteine SH-imidazole hydrogen bond formation hindering protonation of the active site imidazole in the free enzyme. Recent work (Matthews et al., 1977) suggests that such a hydrogen bond may be absent in native STL, at least in the absence of bound substrate (Cruickshank & Kaplan, 1975). If there is no hydrogen bond between serine-221 and histidine-64 in native STL, it is difficult to explain the absence of the normal pK of 7 in SHSTL except by postulating a thiol group–imidazole ring interaction.

Figure 5 also shows that SHSTL has lower k_2/K_s values above the pH 8.45 transition. A possible interpretation of this may be that the transition represents ionization of the active site thiol group to thiolate anion. This was concluded by Neet et al. (1968) who observed this transition in alkylation of the SHSTL thiol group by iodoacetamide. They noted that alkylation is slightly more rapid above this pH than below it. However, two factors suggest another possible cause of the transition observed herein.

The first factor is that STL (Bosshard & Berger, 1974), as well as CHT (Johnson & Knowles, 1966; Smallcombe et al., 1972), binds α-N-acylamino acid anions only below pH 7 where either the active site aspartate (32 in STL) or imidazole (64 in STL) is protonated (Hunkapiller et al., 1973; Markley & Porubcan, 1976). The crystal structure of specific carboxylate anions bound in their productive configuration places the carboxyl group close to the active site serine hydroxyl group (Robertus et al., 1972). Therefore, the charge effect of the aspartate anion could be efficiently transmitted to the area of the serine hydroxyl group. This is presumably one cause of the special reactivity of this group.

The existence of this negative charge near the serine hydroxyl is indicated in the deacylation of acetyl-CHT by the fact that ionization of the strong nucleophile, isonitrosoacetone, which should give an even more nucleophilic anion, totally abolishes the compound's effectiveness (Wedler et al., 1970). This occurs at pH 9 and above and cannot be due to factors unrelated to charge, since uncharged nucleophiles show no such effect (Bender et al., 1964a).

The second factor is that nearby negative charges have strong effects on thiol group ionization constants (Edsall & Wymann, 1958; Flohe et al., 1972). It should be noted that the isoelectric point of STL is in the range of 7.8–9.1 (Matsubara et al., 1965; Ottesen & Spector, 1960) and that the overall net charge of the enzyme molecule must change considerably in the range where the activity transition is observable.

This suggests that the overall charge on the enzyme should affect the equilibrium constants in Scheme I. This scheme incorporates the suggestion of Polgar & Bender (1969) and Polgar et al. (1973) that the thiol group is more likely to form a hydrogen bond or ion pair to the imidazole of histidine-64 than is the hydroxyl group of serine-221. This is due to the larger covalent radius of the sulfur atom.

A perturbation due to isoelectric point is sufficient to explain the effects observed here, since the various species shown in Scheme I must have different catalytic activities toward substrate.

Discussion

There are three primary questions posed by the reactivity of SHSTL. These are (1) whether any site distortions are reflected in SHSTL substrate specificity, (2) whether acylation is the result of normal enzymatic processes, and (3) why deacylations are slower than those in STL.

The first question is addressed both by data presented by Tsai & Bender (1979) and by the chain length–specificity profiles for k_2/K_s, k_{cat}, and K_m presented here. The similarity of the profiles as well as the work of Tsai & Bender (1979) indicates that enzyme–substrate complexes must be very similar in conformation in STL and SHSTL and, thus, indicates that there are no large steric alterations at the active site as a result of the O–S transformation.

Data relating to the second question are given by the known nonreactivity of alkyl ester and amide substrates (Neet et al., 1968; Tsai & Bender, 1979), combined with the good reactivity for a thiolethyl ester (Polgar, 1972). While the data presented in this paper are consistent with the idea that reactivity of SHSTL is due to nucleophilic attack of the sulfur atom on the substrate, they do show that ionization of groups adjacent to the thiol group strongly affects reactivity. The effect is most evident in the pK of 6.08 observed in Figure 5. The pK of 6.08 appears to reflect a sulfur-induced shift of the active site pK of 7.15 observed in acyl-SHSTLs, acyl-STLs, and native STL. This supports the model proposed by Polgar et al. (1973) as a result of their alkylation data on SHSTL Carlsberg, namely, the formation of an imidazole–cysteine ion pair. An ion-pair hypothesis modified to account for possibilities suggested by some NMR studies (Markley & Ibanez, 1978) is also useful in suggesting reasons for the pK of 8.45 since simple thiol group ionization appears to be unlikely at this pH, due to the effect of the neighboring carboxylate anion.

The data in this paper do not provide information concerning the nonreactivity of amides and alkyl esters with SHSTL. However, it might be noted that the increases in substrate specificity observed here are not reflected in SHSTL k_2/K_s values as well as they are reflected in analogous STL values. The K_m chain length dependencies presented here indicate that the least specific substrate studied here has the highest ratio of k_2(SHSTL)/k_2(STL). Therefore, the nonreactivity of specific alkyl esters and amides in the acylation step may be related not only to the type of leaving group but also to their high substrate specificity.

This view is supported by the reactivity of SHSTL toward nonspecific, highly activated anilides (Tsai, 1977), which is similar to that seen in native STL (Bender & Philipp, 1973).

This effect may be due to the size differences between oxygen and sulfur atoms, which may be more critical in more specific substrates. That the oxygen–sulfur size difference has effects on the kinetics of even more nonspecific substrates is indicated by the data of Table I, showing that steric hindrance in the substrate k_2/K_s has significant effects on STL and SHSTL.

Another reason for the lack of SHSTL activity toward nonactivated amides and esters may be only related to differences between oxygen and sulfur nucleophilicities. Jencks & Carriuolo (1960) have shown that oxygen and sulfur nucleophiles show a single pK–reactivity relationship toward NP acetate. If a similar relationship holds for amides, the relatively low pK of the active site thiol group may result in a

lower nucleophilicity than that of the hydroxyl group in the native enzyme.

However, results of various studies indicate that, in substrates with poor leaving groups, breakdown of a tetrahedral intermediate may be rate determining during acylation (Hirohara et al., 1977; Philipp & Bender, 1973; Lucas & Caplow, 1973). It may therefore be more useful to examine oxygen–sulfur effects during breakdown of the tetrahedral intermediate. Acylation studies using oxy- and thioethyl esters with serine proteases indicate that the cysteine thiol group must be a better leaving group than the serine hydroxyl group. In addition, studies on the breakdown of two tetrahedral oxygen and sulfur containing compounds indicate the preferential release of thiols as compared with alcohols (Jencks, 1969; Hershfield & Schmir, 1972; Komiyama & Bender, 1979). Preferential elimination of the cysteine thiol group in a tetrahedral SHSTL–substrate intermediate would cause regeneration of substrate and free enzyme. Thus, acylation of SHSTL by amides may be slow because the cysteine thiol group is preferentially eliminated from a tetrahedral intermediate.

Data relating to the third question posed above, why deacylations are slower in SHSTL than in STL, are given in Figure 2, showing that deacylation–chain length profiles for the two enzymes are similar. *Both enzymes have higher k_3 values than does chymotrypsin.* This pattern is also seen in the hindered substrate, NP trimethylacetate. It is in terms of k_3 that STL and SHSTL show the most similar behavior, both in specificity, pH profile, and solvent deuterium isotope effect (Polgar & Bender, 1969). Since the sulfur atom in the acyl enzyme cannot participate in ionizations, and since preferential elimination of the sulfur atom from a tetrahedral intermediate cannot hinder the reaction, this similarity might be expected. The 10–20-fold reduction in k_3 on O–S transformation may therefore be assigned to effects due to size differences in the oxygen and sulfur atoms.

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