Kinetics of Hydrolysis of *p*-Nitrophenyl Thiolacetate by Chymotrypsin

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Abstract: The kinetics of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl thiolacetate has been compared to that of *p*-nitrophenyl acetate. The oxygen and the thiol ester are hydrolyzed through an identical pathway comprising an enzyme–substrate complex and an acetyl enzyme. The pH dependency and the absolute value of the rate constants for the acylation step are identical within experimental error for both esters from pH 5 to 10. Since thiolate ions are better leaving groups than alcoholate ions, these results strongly suggest that the rate-limiting step of acylation of the enzyme is a bond-making process, thereby necessitating the existence of an additional intermediate, in which the carbonyl carbon most likely assumes a tetrahedral configuration.

The pathway of α-chymotrypsin-catalyzed hydrolysis reactions includes the formation and decomposition of an acyl–enzyme intermediate.[1] Mechanistically, both of these steps have been shown to be substitution reactions at the carbonyl carbon of the substrate, with a serine hydroxyl and water acting as nucleophiles. With few exceptions, nonenzymatic acyl substitution reactions with carbonyl–oxygen fission occur through the formation of an acyl–nucleophile adduct in which the carbonyl carbon assumes a tetrahedral configuration.[2] This tetrahedral intermediate is not directly observable in aqueous media, due to its rapid rate of decomposition. Its intermediacy is, however, demonstrable by the fact that carbonyl–oxygen isotope exchange occurs during hydrolysis.[3] Further indirect evidence for a tetrahedral intermediate in ester and amide hydrolysis comes from the symmetry of the general base catalyzed transesterification reaction,[4] from structure–reactivity relationships,[2] from the unusual pH dependency of thiol ester hydrolysis in acidic media,[5] and from mechanistic studies of the reactions of nucleophiles with analogs of carbonyl derivatives.[6]

A tetrahedral intermediate has also been postulated in α-chymotrypsin-catalyzed reactions, but the experimental evidence for or against its existence is much less compelling. For example, the absence of an observable oxygen isotope exchange during the hydrolysis of *N-trans*-cinnamoyl-α-chymotrypsin[7] could be interpreted as being due either to the asymmetry of the active center or to a one-step acyl-transfer reaction. Similarly, a simultaneous transfer of two protons by general acid–general base catalysis could result in a one-step displacement reaction which would still fulfill the symmetry requirements of the enzyme-catalyzed reaction.[8] Finally, because of the absence of an extensive series of appropriate substrates, studies of structure–reactivity relationships provide only fragmentary evidence for the possible existence of a tetrahedral intermediate.[9]

A much overlooked indirect evidence for the tetrahedral intermediate can be found in the observation that the alkaline hydrolysis of oxygen and thiol esters proceeds by almost identical rates and with identical mechanisms. It is estimated that in a SN2 type reaction an –SR group should be displaced at least 250 times faster than an –OR group.[10,11] Therefore, the identity of the hydrolysis rate of oxygen and thiol esters must mean that the bond-breaking process does not occur in the rate-determining step and thus must occur in a rapid step subsequent to a slow addition of the nucleophile.[12] This conclusion is further supported by the kinetic data on the aminolysis of oxygen and thiol esters.[10]

Essentially the same argument could also be applied to chymotrypsin-catalyzed ester hydrolysis, since preliminary evidence suggests that thiol esters are substrates in this reaction.[13] We chose to study the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl thiolacetate because the acylation reaction of its oxygen analog, *p*-nitrophenyl acetate, is easily measurable and has already been extensively studied.[14–16] In the present paper we intend to show that both the mechanism and the rates of acylation of α-chymotrypsin by *p*-nitrophenyl thiolacetate and *p*-nitrophenyl acetate are identical, thereby providing evidence for the existence of a tetrahedral intermediate on the pathway of the enzyme-catalyzed hydrolysis.

Experimental Section

Materials. Thrice-crystallized α-chymotrypsin and twice-crystallized β-chymotrypsin were Worthington products. Enzyme solutions were prepared and standardized as described previously.[17] *p*-Nitrophenyl acetate (Aldrich Chemical Co.) was recrystallized from chloroform–hexane; mp 79.5–80° (lit.[16] mp 79.5–80°). *p*-

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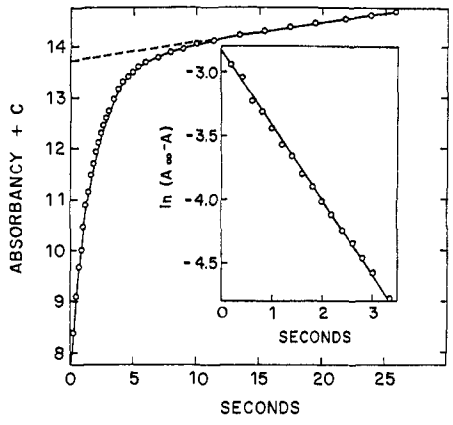

Figure 1. Pre-steady state of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl thiolacetate, 25 ± 0.2°, 0.05 *M* potassium phosphate buffer, 0.2 *M* potassium chloride, and 2.5% acetonitrile, pH 7.07, $[E_0] = 2.07 \times 10^{-6}$ *M*. Absorbancy expressed in units of 10^{-2} AU/2 cm.

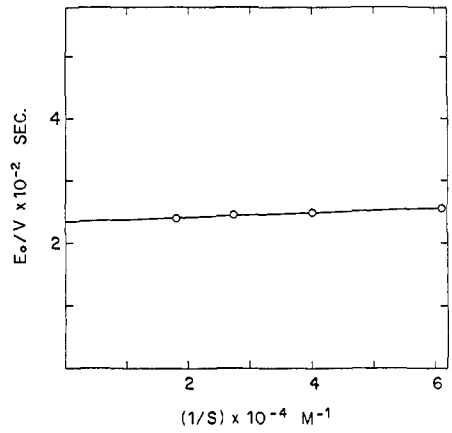

Figure 2. Steady-state hydrolysis of *p*-nitrophenyl thiolacetate by chymotrypsin, 25 ± 0.2°, 0.05 *M* potassium phosphate buffer, 0.2 *M* potassium chloride, 2.5% acetonitrile, pH 7.07, $[E_0] = 2.07 \times 10^{-6}$ *M*.

Nitrothiophenol (Pierce Chemical Co.) was purified by several recrystallizations from cyclohexane; mp 80.1–80.4° (lit.[18] mp 78–79°). All buffers were prepared from glass-distilled water and analytical grade chemicals.

p-Nitrophenyl thiolacetate was synthesized by the dropwise addition of a 20% excess of acetyl chloride to a stirred solution of 10 ml of dry pyridine and 1 g of *p*-nitrothiophenol at 3 ± 3°. After addition was completed, the reaction mixture was allowed to stand for 10 min and was then poured over crushed ice containing enough concentrated HCl to acidify the final mixture. The insoluble product was collected by filtration and recrystallized repeatedly from cyclohexane; mp 82.3–82.6° (lit.[19] mp 82°).

Anal.[20] Calcd for $C_8H_7NO_3S$: C, 48.72; H, 3.58; N, 7.10; S, 16.26. Found: C, 48.50; H, 3.38; N, 7.04; S, 16.18.

Spectra. All spectral measurements were performed with a Cary Model 15 recording spectrophotometer equipped with a thermostated cell holder which maintained a temperature of 25 ± 0.2°. The spectrum of un-ionized *p*-nitrothiophenol was measured in 0.1 *M* HCl; λ_{max} 326 nm, ϵ_{max} 10.49×10^3. The absorption of *p*-nitrothiophenolate ion was measured in 0.1 *M* NaOH; λ_{max} 409 nm, ϵ_{max} 13.93×10^3. At several pH values between 4 and 5 (0.05 *M* acetate buffer, 0.20 *M* KCl), the spectra exhibited well-defined isosbestic points at 273 and 358 nm. The pK_a of *p*-nitrothiophenol calculated from these spectra was 4.47 ± 0.01. The spectra and pK_a of the product derived from the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl thiolacetate were indistinguishable from that of *p*-nitrothiophenol.

Kinetic Measurements. The time dependency of the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl thiolacetate and *p*-nitrophenyl acetate was measured with a Cary Model 15 or a Durrum-Gibson stopped flow spectrophotometer according to methods described previously.[16] All reactions were performed at 25 ± 0.2°. First- and second-order rate constants were determined graphically, using data from at least 90% of the reaction.

In order to supress the reaction of the product, *p*-nitrothiophenol, with a contaminant present in the enzyme preparations, it was necessary to subject the enzyme stock solution to a gel filtration on Sephadex G-25 at pH 4.5 and room temperature. Also the steady-state release of *p*-nitrothiophenol could be complicated by a slow nonenzymatic disappearance of this thiol product. The use of degassed buffers containing 5×10^{-5} *M* EDTA seemed to supress this artifact. Finally, on the time scale of our experiments, even relatively large amounts of *p*-nitrothiophenol did not affect the stability of chymotrypsin.

Results

The kinetics of α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl thiolacetate have been measured by determining the spectral changes at 410 nm associated with the release of *p*-nitrothiophenolate ion. The kinetic experiments were carried out in a variety of conditions, the parameters varied being pH, ionic strength, and the relative concentrations of enzyme and substrate. In conditions where $[E_0] \ll [S_0] \gg K_m$, the amount of *p*-nitrothiophenolate ion produced at the end of the presteady state always equaled the concentration of α-chymotrypsin in the reaction mixture as determined by titration with *N-trans*-cinnamoylimidazole. Therefore, *p*-nitrophenyl thiolacetate can be used as a titrant in determining the normality of chymotrypsin solutions.

In a first set of experiments we used substrate in great excess with respect to enzyme. A typical time dependency of product formation is shown in Figure 1. As in the case of the hydrolysis of the oxygen ester[14] an initial rapid release of *p*-nitrothiophenol is observed followed by a slow, linear steady-state hydrolysis. From the latter portion of the curve, the kinetic constants of the steady state have been calculated after correcting for the spontaneous hydrolysis of the substrate. The rate of the enzymatic reaction, v, was found to obey a Michaelis–Menten-type equation (eq 1).

$$v = \frac{k_{cat}[E_0][S_0]}{K_m + [S_0]} \quad (1)$$

The values of the constants k_{cat} and K_m at pH 7.49 have been determined by plotting $[E_0]/v$ *vs.* $1/[S_0]$ (Figure 2) and are compared with the corresponding values for the oxygen ester in Table I. In complete analogy with the oxygen ester, deviations from linearity were observed in these plots at high substrate concentrations, presumably due to substrate activation.

In order to confirm that an acetyl–enzyme is formed in the course of the reaction, α-chymotrypsin was treated with *p*-nitrophenyl thiolacetate at pH 5.0 and the reaction product purified by gel filtration on Sephadex G-25 fine at pH 4.0. In this way it was possible to obtain a 90–95% inactivated enzyme which, when incubated at higher pH values, slowly regained activity toward *p*-nitrophenyl benzyloxycarbonyl-L-tyrosinate. This reactivation followed first-order kinetics and the deacylation rate constants at two pH values were found to be identical with the corresponding rate constants for

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(20) Microanalyses performed by Microtech Lab., Inc., Skokie, Ill.

Table I. Kinetic Parameters of the α-Chymotrypsin-Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate and *p*-Nitrophenyl Thiolacetate[a]

	pH	NPA	NPTA
$k_{cat} \times 10^3$ sec^{-1}	7.48	4.17[b]	4.26
$K_m \times 10^6$ M	7.48	1.58[b]	1.46
k_{cat}/K_m, M^{-1} sec^{-1}	7.48	3110[b]	2940
$k_3 \times 10^3$ sec^{-1}	7.06	2.6[b]	2.9
	6.30	0.64[b]	0.60
k_2 sec^{-1}	7.07	3.6,[b] 2.2[c]	2.9[d]
	5.04	0.13[c]	0.11
k_2/K_s, M^{-1} sec^{-1}	7.07	2940[c]	2450[d]
	5.04	79[c]	178
$K_s \times 10^3$ M	7.07	1.47[c]	1.20[d]
	5.04	1.74[c]	0.67

[a] Buffers contained 1.6% acetonitrile except where noted, I = 0.25 M, t = 25°. [b] From the data of Kézdy and Bender.[16] [c] From the data of Bender, *et al.*[15] [d] Buffers contained 2.5% acetonitrile.

Table II. Second-Order Rate Constants for the Acylation of α-Chymotrypsin by *p*-Nitrophenyl Acetate and *p*-Nitrophenyl Thiolacetate, $[S_0] \gg [E_0]$[a]

Buffer	pH	$(k_2/K_s)_{NPTA}$, M^{-1} sec^{-1}	$(k_2/K_s)_{NPA}$, M^{-1} sec^{-1}
Part 1, I = 0.005 M			
Acetate	4.47	40	
Acetate	4.93	117	
Acetate	5.02		72
Acetate	5.43	278	254
Acetate	5.80	560	
Acetate	5.93		500
Phosphate	6.48	1360	1340
Phosphate	7.15	2500	2100
Phosphate	7.41	2340	2450
Phosphate	7.52	2670	
Phosphate	7.50		2630
Phosphate	7.69	2970	2930
Veronal	8.20	3000	2850
Veronal	8.64	2570	2370
Carbonate	8.98	2080	1990
Carbonate	9.65	1140	1060
Part 2, I = 0.25 M			
Acetate	4.15	59	
Acetate	4.54	204	
Acetate	4.98	445	
Acetate	5.32	514	
Acetate	5.88	627	
Phosphate	6.48	1030	
Phosphate	6.68	1470	
Phosphate	7.18	2827	
Phosphate	7.33	3410	
Veronal	7.61	3200	
Veronal	7.95	3530	
Veronal	8.24	3260	
Veronal	8.44	3540	
Carbonate	8.92	2720	
Carbonate	9.48	1230	
Carbonate	9.97	717	

[a] Buffers contain 1.6% acetonitrile, t = 25°, $[E_0] = 3.5 \times 10^{-6}$–$2 \times 10^{-5}$ M, $[S_0] = 4 \times 10^{-5}$–1×10^{-4} M.

authentic acetylchymotrypsin (Table I). These results establish that the pathway of the α-chymotrypsin-catalyzed hydrolysis of the thiol ester is identical with the pathway of the oxygen ester. This pathway is represented by eq 2.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (2)$$
$$\qquad\qquad\qquad + P_1$$

The initial portion of the curve shown in Figure 1 represents the pre-steady-state accumulation of acetyl-

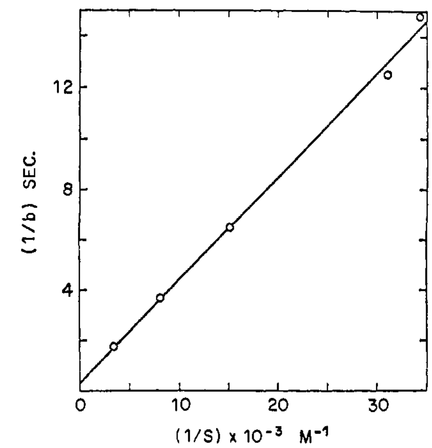

Figure 3. Acylation of α-chymotrypsin by *p*-nitrophenyl thiolacetate, 25 + 0.2°, 0.05 M potassium phosphate buffer, 0.2 M potassium chloride, 2.5% acetonitrile, pH 7.07, $[E_0] = 2.07 \times 10^6$ M or 1.86×10^{-6} M.

chymotrypsin (ES′). A good first-order plot yielding an apparent rate constant, b, was obtained when the pre-steady-state portion of the curve was analyzed according to methods described previously[16] (inset, Figure 1). From a plot of $1/b$ *vs.* $1/[S]$ (Figure 3) k_2 and K_s could be calculated.[16] These values at two pH's are given in Table I together with the literature values for the oxygen ester.

Due to the high value of K_s and the poor solubility of the thiol ester, it was not practical to measure k_2 and K_s independently at many pH's. Therefore, we carried out most of our experiments in conditions where substrate concentration was much lower than K_s and thus obtained the second-order rate constant k_2/K_s.[16] For comparison, the second-order acylation rate constant for the oxygen ester was determined under identical conditions. The results of these experiments at several pH's and two ionic strengths are reported in Table II.

In a second set of experiments the second-order rate constants of acylation have been determined in conditions where enzyme and substrate concentrations were comparable, and both less than K_s but greater than K_m.[16] In these conditions the reaction obeys second-order kinetics (Figure 4), and one can directly determine k_2/K_s. These values at several pH's are reported in Table III. The data obtained under first-

Table III. Second-Order Rate Constants for the Acylation of α-Chymotrypsin by *p*-Nitrophenyl Thiolacetate, $[S_0] \gtrless [E_0]$[a]

Buffer	pH	k_2/K_s, M^{-1} sec^{-1}
Acetate	4.12	65
Acetate	4.63	248
Acetate	5.05	452
Acetate	5.33	464
Acetate	5.98	795
Phosphate	6.12	891
Phosphate	6.50	1315
Phosphate	6.58	1445
Phosphate	6.95	2405
Phosphate	7.12	2560
Phosphate	7.49	3170
Phosphate	7.88	3620

[a] Buffers contain 1.6% acetonitrile, I = 0.25 M, t = 25°, $[E_0] = 3.5 \times 10^{-5}$–$6 \times 10^{-5}$ M, $[S_0] = 2.6 \times 10^{-5}$–$5.1 \times 10^{-5}$ M.

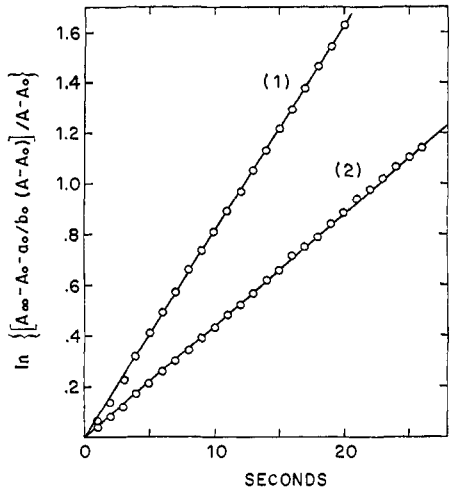

Figure 4. Acylation of α-chymotrypsin by *p*-nitrophenyl thiolacetate, 25 ± 0.2°, 0.05 *M* potassium phosphate buffer, 0.2 *M* potassium chloride, 1.6 acetonitrile, $[E_0] = 5.99 \times 10^{-5}$ *M*, $[S_0] = 2.60 \times 10^{-5}$ *M*; curve 1, pH 6.95; curve 2, pH 6.50.

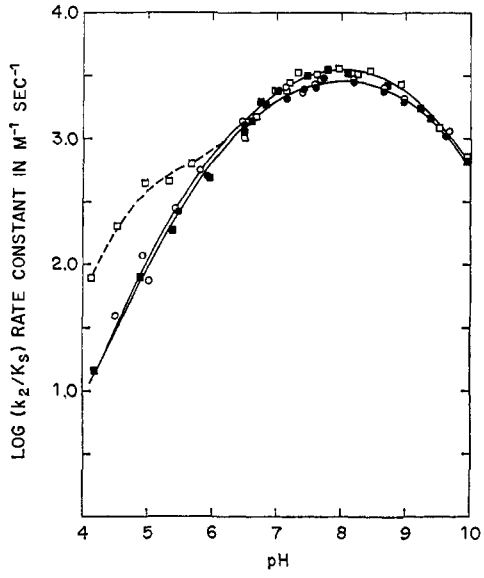

Figure 5. pH dependency of the acylation of α-chymotrypsin by *p*-nitrophenyl acetate and *p*-nitrophenyl thiolacetate: *p*-nitrophenyl thiolacetate, $I = 0.005$ *M* (○); *p*-nitrophenyl acetate, $I = 0.005$ *M* (●); *p*-nitrophenyl thiolacetate, $I = 0.25$ *M* (□); and *p*-nitrophenyl acetate, $I = 0.05$–0.10 *M* (■).[15]

and second-order conditions at several ionic strengths are summarized in Figure 5.

At low ionic strength all the experimental points for both esters lie on a smooth bell-shaped curve which can be described by the expression

$$k_2/K_s = \frac{(k_2/K_s)_{lim}}{1 + H/K_1 + K_2/H} \quad (3)$$

By graphical curve fitting one can determine the best values for the parameters in eq 3. These are reported in Table IV along with the other pH-independent rate constants for the chymotrypsin-catalyzed hydrolysis of the oxygen and thiol ester. As can be seen from Table IV and Figure 5, the second-order rate constants for the acylation of α-chymotrypsin by the oxygen and the thiol ester are consistently higher at ionic strengths of 0.1–0.25 *M* than at 0.005 *M*.

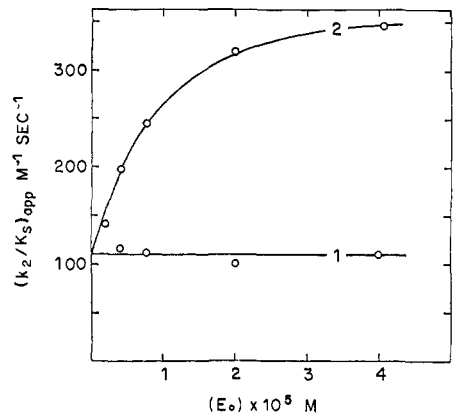

Figure 6. Acylation of α-chymotrypsin by *p*-nitrophenyl thiolacetate, 25 ± 0.2°, $[S_0] = 1.00 \times 10^{-4}$ *M*, 1.6% acetonitrile, and 0.005 *M* potassium acetate buffer, pH 5.05 (curve 1), and 0.05 *M* potassium acetate buffer, 0.2 *M* potassium chloride, pH 5.05 (curve 2).

These differences are relatively trivial, and no observable shift in the ionization constants seems to occur. However, some of the experimental values for k_2/K_s determined for the thiol ester at high ionic strength and pH values below 6 deviate markedly from the theoretical bell-shaped curve (Figure 5). Further investigation revealed that this abnormal behavior was enzyme-concentration dependent. Figure 6 shows that at pH 5.05 the second-order rate constant is independent of the enzyme concentration at low ionic strength (curve 1), while at high ionic strength (curve 2) increasing the enzyme concentration increases the magnitude of the apparent rate constant. At high enzyme concentrations a saturation phenomenon is observed. Most importantly, at very low enzyme concentration, the high and low ionic strength experiments yielded identical rate constants. We therefore strongly feel that the enzyme concentration dependent increase of the rate constant has no intrinsic bearing on the mechanism of acylation of the enzyme by the thiol ester. Since this phenomenon occurs at pH's and ionic strengths where dimerization of the enzyme is most pronounced[21] it might be due to aggregation of the enzyme. Furthermore, β-chymotrypsin in no way ex-

Table IV. Kinetic Parameters of α-Chymotrypsin-Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate and *p*-Nitrophenyl Thiolacetate

	NPA	NPTA
$(k_{cat})_{lim} \times 10^3$ sec^{-1}	6.8[a]	6.9[b]
$K_m \times 10^6$ *M*	1.59[a,c]	1.46[d]
k_{cat}/K_m	2640[a,c]	2940[d]
$(k_2)_{lim}$, sec^{-1}	4.8,[a] 4.55[e]	4.52[f,g]
$K_s \times 10^3$ *M*	1.47[e]	1.20
$(k_2/K_s)_{lim}$, M^{-1} sec^{-1}	4060,[a] 3940[e]	3770[g]
$(k_2/K_s)_{lim}$, M^{-1} sec^{-1}	3350[h]	3350[h]
pK_1	6.85[a]	6.89[g]
pK_2	9.04[a]	9.01[g]

[a] From the data of Kézdy and Bender.[16] [b] Calculated from data at pH 7.48 assuming pK = 7.28.[16] [c] pH 7.8, $I = 0.25$ *M*. [d] pH 7.48, $I = 0.25$ *M*. [e] From the data of Bender, *et al.*[15] [f] Calculated from the values for $(k_2/K_s)_{lim}$ and K_s presented in the table. [g] $I = 0.25$ *M*. [h] $I = 0.005$ *M*.

(21) F. J. Kézdy and M. L. Bender, *Biochemistry*, 4, 104 (1965).

Table V. Second-Order Rate Constants for Acylation of α- and β-Chymotrypsin by *p*-Nitrophenyl Acetate and *p*-Nitrophenyl Thiolacetate[a]

Enzyme	$[E_0] \times 10^5$, M	I, M	pH	$(k_2/K_s)_{NPTA}$, M^{-1} sec^{-1}	$(k_2/K_s)_{NPA}$, M^{-1} sec^{-1}
β-CHT	4.24	0.005	5.04	103	
	4.24	0.25	5.06	105	
	2.98	0.25	5.02		72
	2.98	0.25	7.00	3185	3016
α-CHT	3.80	0.25	5.05	365	78
	(0)[b]	0.25	5.05	(110)[b]	
	4.02	0.005	5.04	110	
	3.53	0.25	7.10		2700
	6.05	0.25	6.95	2800	

[a] Buffers contain 1.6% acetonitrile, $t = 25°$, $[S_0] = 2.5 \times 10^{-5}$–$3.3 \times 10^{-4}$ M. [b] Graphical extrapolation to zero enzyme concentration.

hibits this abnormal behavior (Table V). At present we are unable to explain why the abnormal enzyme concentration dependency observed under certain conditions for the thiol ester is not seen with the oxygen ester.

Discussion

The only previous report on the hydrolysis of thiol esters by chymotrypsin was based on the qualitative observation that, in the presence of enzyme, ethyl *N*-acetyl-*dl*-thiolphenylalaninate yielded a volatile compound which gave a positive nitroprusside test.[13] The present results establish that chymotrypsin does indeed catalyze the hydrolysis of thiol esters. The pathway for the hydrolysis of *p*-nitrophenyl thiolacetate and its oxygen analog is identical since the same acetylchymotrypsin is formed as an intermediate. Furthermore, the rate constants for these two reactions are essentially the same over a wide range of pH values, suggesting that the mechanism of the rate-limiting step in the acylation reaction is also identical for both substrates.

The facile reaction of the thiol ester with chymotrypsin confirms the general observation that the enzyme possesses no specificity for the atom displaced during acylation. Thus the enzymatic reactivity of a series of substrates possessing the same acyl group and a variety of leaving groups, such as acid chlorides, anhydrides, esters, amides, acylimidazoles, and even thiol esters, is determined solely by the intrinsic reactivity of these compounds toward any oxynucleophile.

The quasiidentity of the rate constants for acylation of chymotrypsin by oxygen and thiol esters indicates that cleavage of the carbon–oxygen and carbon–sulfur bonds cannot occur in the rate-determining step. Since –SR is at least 250 times a better leaving group than –OR, the thiol ester should react much faster if the leaving group was eliminated in the same step where bond formation with the enzyme occurs. The carbon–oxygen and carbon–sulfur bonds must, therefore, be broken in a second fast step, thus necessitating the existence of an intermediate. The fast bond-breaking step must occur after the slow step since no detectable amount of intermediate accumulates during the reaction. Given the existence of two steps, the simplest possible pathway for acylation is obtained when the slow step is identified as being the formation of a bond between the carbonyl carbon and the serine oxygen, yielding a tetrahedral intermediate (TI). *A priori*, the experimentally observed rate constant of acylation, k_2, could then be a combination of the rate constants defined by the following equation (eq 4).

$$ES \underset{k_{-1}}{\overset{\text{slow } k_1}{\rightleftharpoons}} TI \xrightarrow{\text{fast } k_{II}} ES' + P_1 \qquad (4)$$

Applying the steady-state assumption to the concentration of TI, the following relationship is obtained.

$$k_2 = \frac{k_I k_{II}}{k_{-I} + k_{II}} \qquad (5)$$

The failure to observe the tetrahedral intermediate requires that $k_{II} \gg k_I$. The quasiidentity of k_2 for the oxygen and thiol esters requires also that $k_{II} \gg k_{-I}$. This is well in agreement with the behavior of the two types of esters toward alkaline hydrolysis.[22]

Nitrogen nucleophiles are known to react much faster with thiol esters than with their oxygen counterparts, the rate-limiting step being addition of the nucleophile.[10] Our results thus strongly argue against the occurrence of any mechanism where the imidazole of histidine-57 would be the primary nucleophile and the hydroxyl group of serine-195 the final acyl acceptor. Finally, the well-established similarity of the acylation and deacylation reactions requires the occurrence of a tetrahedral intermediate in the deacylation step also.

The evidence described in this report for the occurrence of a tetrahedral acyl–nucleophile adduct is of necessity indirect because of the expected instability of such an intermediate. Nevertheless, the arguments presented make use of only a minimum number of assumptions and therefore constitute one of the strongest evidences for the inclusion of a tetrahedral intermediate on the pathway of chymotrypsin-catalyzed hydrolysis reactions.

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