The Elastase-Catalyzed Hydrolysis of *p*-Nitrophenyl Trimethylacetate[1]

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Abstract: The hydrolysis of excess *p*-nitrophenyl trimethylacetate by the enzyme elastase is a two-step process consisting of a fast initial liberation of *p*-nitrophenol followed by a slower linear turnover. The initial rate of the former reaction shows pure first-order dependence on substrate concentration without preequilibrium complex formation while the rate of the latter reaction shows a Michaelis–Menten behavior. The over-all reaction is interpreted in terms of an acyl-enzyme intermediate whose deacylation is rate determining in the over-all reaction. Further support for an acyl-enzyme intermediate includes: (1) maximum turnover rates for the *o*- and *p*-nitrophenyl trimethylacetates are the same whereas their basic hydrolysis rates are 4.9-fold different. (2) Tris(hydroxymethyl)aminomethane and methanol accelerate the enzymic hydrolysis rate, an effect consistent with an accelerating effect on the rate-determining deacylation. The pH dependence of the initial rate of the presteady-state (acylation) reaction is a sigmoid curve with an inflection point at pH 6.7. The steady-state (deacylation) reaction exhibits a bell-shaped pH–rate profile with apparent pK's of 6.7 and 10.5. The pK of 6.7, in each instance, is interpreted as indicative of the catalytic participation by a neutral imidazole group. The substantial deuterium oxide solvent isotope effects, found both in acylation and deacylation reactions, are interpreted as evidence for general base catalysis.

Elastase is a pancreatic[2] proteolytic[3] enzyme which shows many similarities to the enzymes chymotrypsin and trypsin. Elastase is reported to catalyze the hydrolysis of casein, hemoglobin, and ethyl N-acetyl-L-tyrosinate[4] as well as peptide linkages involving phenylalanine, tyrosine, and glutamic and aspartic acids.[5] Like chymotrypsin and trypsin, elastase is irreversibly inhibited by diisopropyl phosphorylfluoridate,[6] the inhibition occurring at a unique serine residue. The amino acid sequence surrounding this residue is very similar to that found in chymotrypsin and trypsin as is the sequence of amino acids in the histidine region of these enzymes.[6-9] Elastase, chymotrypsin, and trypsin also react with diethyl *p*-nitrophenyl phosphate in a stoichiometric reaction leading to a completely inactive enzyme.[10] Thus elastase is a member of the family of enzymes known as the "serine proteinases" [11] and may show mechanistic similarity to chymotrypsin and trypsin catalyses. In order to probe this similarity the elastase-catalyzed hydrolysis of *p*-nitrophenyl trimethylacetate has been investigated.

Experimental Section

Materials. *p*-Nitrophenyl trimethylacetate was synthesized previously.[12] It was recrystallized twice from ethanol, mp 93.5–94.5°; lit.[13] 94–95°. Upon basic hydrolysis it liberated 100% of the theoretical amount of *p*-nitrophenol.

o-Nitrophenyl trimethylacetate was prepared by Dr. J. K. Stoops in this laboratory from trimethylacetyl chloride and *o*-nitrophenol in dry pyridine, bp 90° (0.2 mm). *Anal.* Calcd for $C_{11}H_{13}NO_4$: C, 59.65; H, 6.06; N, 6.24. Found: C, 59.19; H, 5.87; N, 6.27. The ultraviolet spectrum of the hydrolysis products corresponds to that of *o*-nitrophenol.

Stock substrate solutions were made from Eastman Spectro Grade acetonitrile which had been distilled several times from P_2O_5 and finally from K_2CO_3. Methanol was a Fisher certified reagent which was used without further purification. Buffers were made from reagent grade chemicals according to a recipe to give a specific pH at $I = 0.05$ with no organic solvent unless otherwise stated.[14] All pH's recorded were measured on a Radiometer 4C pH meter and it was assumed that the acetonitrile (maximally 7.6% (v/v)) had a negligible effect on the glass electrode reading. The water was glass distilled in a Corning AG-2 still after passing through an Amberlite MB 3 resin and a cationic exchanger. Deuterium oxide was a General Dynamics product containing 99.7 atom % deuterium. The minimum percentage deuterium in any reaction mixture in which the kinetic solvent isotope effect was measured was 96.5%. pD was determined from the pH meter by correcting the pH reading according to the equation: pD = pH-meter reading +0.4.[15]

Both crystalline elastase and electrophoretically purified elastase were obtained from the Worthington Biochemical Corp. The former preparation dissolves readily in 0.05 *M* sodium citrate, pH 4.6, and in 0.05 *M* potassium phosphate, pH 8.0, but not in plain water. Upon storage for several months in the refrigerator the crystalline enzyme changed to a gray amorphous mass insoluble in any of these solvents but it could be solubilized in pH 4.7 calcium acetate solution. On the other hand, the electrophoretically purified elastase dissolved readily in pH 4.7 acetate buffer. The latter preparation is a considerably purer preparation than the former which contains at least three enzymatically active components plus other enzymatically inactive materials. A further purification of the electrophoretically purified elastase was carried out by the means of column chromatography and will be the subject of a separate communication.[16]

Spectra. The ester hydrolyses were followed spectrophotometrically by the liberation of nitrophenol. A summary of the spectral data is given in Table I.

(1) This research was supported by grants from the National Institutes of Health.

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Table I. Summary of Spectral Data[a]

Compound	pK	Wavelength, mμ	Molar extinction coefficient, M^{-1} cm^{-1}	Conditions
p-Nitrophenyl trimethylacetate		347.5[b]	4,840[b]	1.06% (v/v) acetonitrile–water–0.1 *N* NaOH
p-Nitrophenolate	7.04	400 (max)	18,300	1.64% (v/v) acetonitrile–water–0.05 *N* KOH
p-Nitrophenol	7.04	318 (max)	9,560	0.48% (v/v) acetonitrile–water–0.01 *N* HCl
p-Nitrophenolate	7.11	400 (max)	18,600	7.6% (v/v) acetonitrile–water–phosphate, I = 0.05
p-Nitrophenol	7.11	400	140	7.6% (v/v) acetonitrile–water–phosphate, I = 0.05
o-Nitrophenolate	7.07	415 (max)	4,500	3.2% (v/v) acetonitrile–water, 0.1 *M* Tris
o-Nitrophenol	7.07	415	318	3.2% (v/v) acetonitrile–water, 0.1 *M* Tris

[a] All measurements were made with a Cary 14 spectrophotometer at 25°. [b] The change in absorbance coefficient was observed upon hydrolysis of the compound directly in the cell.

Kinetics. Kinetics were measured in a Cary 14 recording spectrophotometer containing a thermostated cell compartment. The pH was routinely measured at the conclusion of each kinetic run since both the activity of the enzyme and the absorption coefficient of the product were strongly pH dependent. In the hydrolysis of *p*-nitrophenyl trimethylacetate the liberation of *p*-nitrophenol was usually observed at 400 mμ. In general, it was necessary to correct the gross rate of hydrolysis for the spontaneous hydrolysis of substrate. The cell containing a solution of buffered substrate was thermostated in the cell compartment of the spectrophotometer for 10–15 min before each kinetic run. The highest substrate concentration used was 1.15×10^{-4} *M*. To obtain this concentration in 3.2% (v/v) acetonitrile–water, it was often necessary to add the aqueous buffer to the substrate rather than *vice versa* in order to prevent precipitation. Stock enzyme was added last to the cuvette on the flattened end of a glass stirring rod. Recording was recommenced within 10 sec or less. The concentration of enzyme solutions was determined by titration of the reactive sites with diethyl *p*-nitrophenyl phosphate.[10]

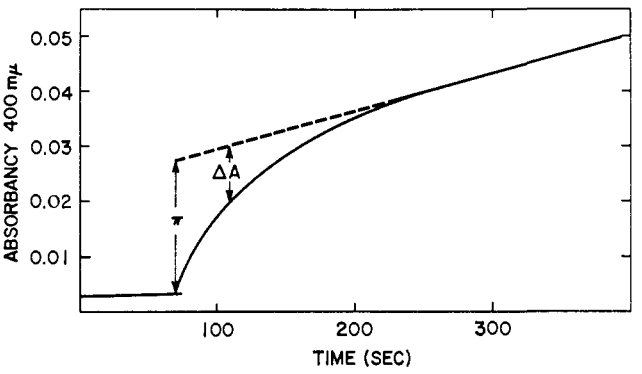

Figure 1. The reaction between elastase (1.86×10^{-6} *M*) and *p*-nitrophenyl trimethylacetate (1.03×10^{-4} *M*) at pH 7.9.

The kinetics of the reaction between elastase and a large excess of *p*-nitrophenyl trimethylacetate were analyzed in terms of eq 1

$$E + S \xrightarrow{k_{II}} ES \xrightarrow{k_3} E + P_2 \quad (1)$$
$$\quad\quad\quad\quad\quad\quad + \quad\quad\quad\quad\quad\quad + P_1$$

where E represents enzyme, S represents substrate, ES represents acyl-enzyme, P_1 represents *p*-nitrophenol, and P_2 carboxylic acid.[17] The reaction consists of a fast initial liberation of *p*-nitrophenol followed by a slow linear hydrolysis (Figure 1). These two phases of the reaction are termed the presteady state and steady state, respectively. The former was analyzed by plotting the logarithm of the difference between the observed curve and an extrapolated portion of the steady-state line and obtaining a conventional first-order kinetic plot. The observed first-order rate constant, b, is related to the microscopic rate constants of eq 1 by eq 2 where

$$b = k_{II}[S]_0 + k_3 \quad (2)$$

k_{II} is the second-order rate constant pertaining to the initial rate of the presteady-state reaction. It is obtained by dividing the initial rate by the concentrations of enzyme and substrate. The initial rate is estimated by making a semilogarithmic plot of the slope of the experimental curve *vs.* time and extrapolating to time zero.

$$k_{II} = \text{initial rate}/[E]_0[S]_0 \quad (3)$$

The steady-state kinetics were analyzed in terms of the mechanism of eq 1 by using eq 4 which is analogous to the usual Michaelis–

$$v = k_3[E]_0[S]_0/([S]_0 + k_3/k_{II}) \quad (4)$$

Menten equation. By making a double-reciprocal (Lineweaver–Burk) plot of $1/v$ *vs.* $1/[S]_0$ it is possible to obtain $V_{max} = k_3[E]_0$, the maximal turnover velocity, and $K_m (= k_3/k_{II})$ from the intercept and slope/intercept of such a plot. The data for a single Lineweaver–Burk plot was obtained in either of two ways: (1) the linear steady-state rates of several reactions using different initial substrate concentrations were measured for a fraction of the total extent of reaction; or (2) a single reaction was observed throughout its entirety, and the rates represented by the slope of experimental cords were plotted with the substrate concentrations corresponding to that point in the reaction.

The kinetics of the basic hydrolysis of the nitrophenyl esters were measured under pseudo-first-order conditions with a large excess of hydroxide ion. At least three different hydroxide ion concentrations were used. Second-order rate constants for the basic hydrolysis were determined from the slope of the plot of the observed first-order rate constant *vs.* the hydroxide ion concentration. Hydroxide ion concentration was calculated from the pH-meter reading and the ion product of water.

Results

The elastase-catalyzed hydrolysis of *p*-nitrophenyl trimethylacetate occurs *via* a two-step reaction in which an initial fast exponential liberation of *p*-nitrophenol is followed by a slower linear liberation. The schematic diagram of the reaction is shown in Figure 1. The initial exponential liberation of *p*-nitrophenol is attributed to a presteady-state acylation reaction while the linear reaction is attributed to a steady-state turnover reaction, the slope of which is greater than that of the spontaneous hydrolysis. The turnover reaction

(17) The mathematical expressions used in this kinetic analysis are derived in the Appendix.

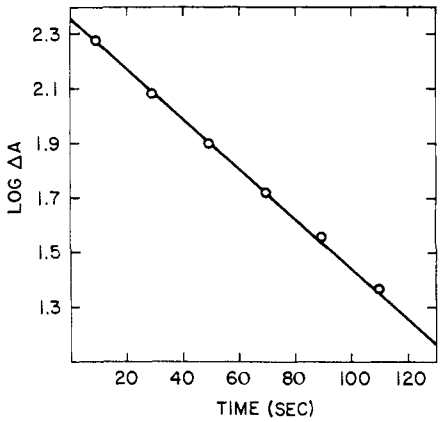

Figure 2. First-order plot of the presteady state of the reaction shown in Fgure 1.

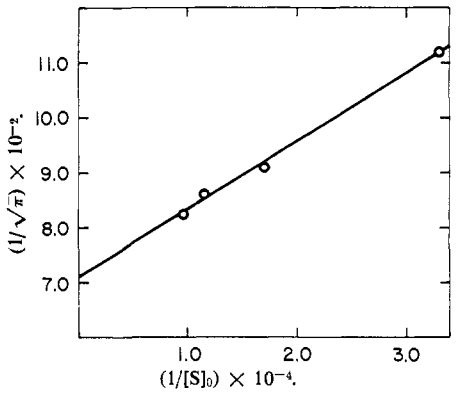

Figure 3. Titration of elastase with *p*-nitrophenyl trimethylacetate. Data of Table III.

indicated that deacylation proceeds at an appreciable rate. The kinetic data are therefore best considered in terms of the pathway of eq 1. The effects of varying enzyme and substrate concentration on the rate of *p*-nitrophenol release are consistent with this scheme. The liberation of *p*-nitrophenol during the presteady-state reaction is a first-order process. A typical kinetic plot using the logarithm of ΔA (Figure 1) *vs.* time is shown in Figure 2. The first-order rate constant obtained is b of eq 2 (see Experimental Section).

The effect of varying the enzyme concentration on various kinetic parameters is shown in Table II. Over a tenfold range b is independent of enzyme concentration; v, the steady-state rate, is directly proportional to the enzyme concentration; and π, the "burst" of *p*-nitrophenol determined by extrapolating the steady-state portion of reaction to zero time (Figure 1), is directly proportional to the enzyme concentration.

Table II. The Effect of Enzyme Concentration on the Reaction with *p*-Nitrophenyl Trimethylacetate[a]

$[E]_0$[b] $\times 10^6$ M	$b \times 10^2$ sec^{-1}	$\pi \times 10^6$ M	$v \times 10^9$ M sec^{-1}
0.65	2.0 ± 1.0	0.477	1.33
1.61	1.7 ± 0.3	1.14	2.76
1.61		1.20	3.18
3.20	1.42 ± 0.05	2.18	5.60
3.20		2.29	5.81
6.31	2.2 ± 0.8	4.40	10.6
6.31		4.63	10.0

[a] 25°, $[S] = 1.15 \times 10^{-4}$ M. 3.18% (v/v) acetonitrile–water pH 7.9, phosphate, $I = 0.05$; elastase 5691/923. [b] Determined by titration with diethyl *p*-nitrophenyl phosphate.

In the experiments reported in Table II the enzyme concentration was determined first by titration with diethyl *p*-nitrophenyl phosphate. It is interesting to note that the burst of *p*-nitrophenol produced in the acylation with *p*-nitrophenyl trimethylacetate is systematically less than the enzyme concentration. The relationship shown in eq 5 between π and $[E]_0$ is derived in the Appendix from the mechanistic scheme of eq 1. The quantity on the right-hand side of eq 5 may

$$\frac{\pi}{[E]_0} = \left(\frac{k_{II}[S]_0}{k_{II}[S]_0 + k_3}\right)^2 \qquad (5)$$

be calculated from the data of Table II and is found to be 0.77; the average value of the ratio on the left from Table II is 0.715. Thus it appears that eq 5 adequately describes the nonstoichiometry of the burst as being due to a deacylation (k_3) proceeding at an appreciable rate. This is in contrast to the reaction of elastase with diethyl *p*-nitrophenyl phosphate which was used for titration of the enzyme.[10] Equation 5 can be considered to be an alternative form of eq 16, published previously.[10]

Table III. The Effect of Substrate Concentration on the Reaction between Elastase and *p*-Nitrophenyl Trimethylacetate[a]

$[S]_0 \times 10^5$ M	$k_{II} \times 10^{-2}$[b] M^{-1} sec^{-1}	$\pi \times 10^9$[c] M	$V \times 10^9$[d] M sec^{-1}	$b \times 10^2$ sec^{-1}
10.28	1.95	1.480	4.500	2.12
8.571	1.75	1.344	4.440	1.87
5.811	2.20	1.215	4.084	1.40
3.000	2.06	0.797	3.505	0.98

[a] $[E]_0 = 1.82 \times 10^{-6}$ M, 5691/923, pH 7.9 Tris, $I = 0.05$, 25.1°, 0.81% (v/v) acetonitrile–water. [b] k_{II} = initial rate/$[E]_0[S]_0$. [c] Size of burst. [d] Rate of (linear) steady state.

The effect of varying the substrate concentration on the kinetics is presented in Table III. The burst π, the steady-state rate, and the presteady-state rate constant b all increase with increasing substrate concentration. This behavior is explained satisfactorily by the mechanism of eq 1.

At a given enzyme concentration the relation between π and the substrate concentration is given by eq 6 (see Appendix). The data of Table III are plotted

$$1/\sqrt{\pi} = 1/\sqrt{[E]_0} + k_3/(k_{II}\sqrt{[E]_0}[S]_0) \qquad (6)$$

according to eq 6 in Figure 3. The intercept gives a value of 1.97×10^{-6} M for $[E]_0$ which compares reasonably well with 1.82×10^{-6} M from titration with diethyl *p*-nitrophenyl phosphate. This agreement constitutes additional evidence that the reaction of elastase with diethyl *p*-nitrophenyl phosphate provides a valid titration. Figure 3 can be used to make an additional check for internal consistency of this analysis. The quotient k_3/k_{II} can be derived from Figure 3 in two ways. The slope can either be divided by the intercept or by $1/[E]_0^{1/2}$ calculated from the results of the diethyl *p*-nitrophenyl phosphate titration. The values of the quotient so obtained are 1.73 and 1.66 $\times 10^{-5}$, respectively, while the ratio of the actual rate constants is 1.42×10^{-5}.

Any acceptable mechanism must successfully explain the variation of b, the observed first-order rate constant of the presteady-state reaction, with substrate concentration. The mechanism of eq 1 predicts a linear relationship between b and the substrate concentration as indicated in eq 2. This relationship is borne out by the data in Table III.

The steady-state rate data are analyzed in terms of eq 4, which is derived from eq 1. The slope and intercept of a double-reciprocal (Lineweaver-Burk) plot give k_{II} and k_3, respectively. The quantity analogous here to the Michaelis constant, K_m, is a ratio of rate constants (k_3/k_{II}) rather than a binding constant. The steady-state rate data of Table III give such a plot.

An additional check on the validity of the mechanism of eq 1 is provided by comparing the second-order rate constants for the presteady state and steady state. The average of the four values of k_{II} from Table III is $2.00 \times 10^2\ M^{-1}\ \text{sec}^{-1}$ while the figure derived from the slope of the Lineweaver-Burk plot of the same data is $1.94 \times 10^2\ M^{-1}\ \text{sec}^{-1}$.

It is appropriate to point out that the mechanism proposed here, involving an acyl-enzyme without preequilibrium complex formation, is different from that which has been used previously to explain burst reactions of nitrophenyl esters with proteolytic enzymes. The following is a mechanism proposed originally[18] and subsequently[19] for the reaction of *p*-nitrophenyl acetate with chymotrypsin.

$$E + S \underset{}{\overset{K}{\rightleftharpoons}} ES \longrightarrow ES' \overset{k_3}{\longrightarrow} E + P_2 \qquad (7)$$
$$+ P_1$$

This mechanism differs from eq 1 by the inclusion of the preequilibrium formation of an adsorptive complex before acylation. It is often assumed that such a (Michaelis) complex is a common feature of enzyme reactions. The present data can be analyzed in terms of eq 7 by deriving k_3 from the maximal velocity of the steady state and determining k_2 and K_S from the presteady state. In so doing the effect of substrate concentration on the observed presteady-state rate constant b must be considered. This is accomplished by the relationship[19]

$$b = \frac{(k_2 + k_3)[S]_0 + K_S k_3}{K_S + [S]_0} \qquad (8)$$

The data can be treated without making any assumptions regarding the relative magnitude of the constants by rearranging the above expression in the following way.

$$\frac{([S]_0/k_{cat}[E]_0) + (K_s/k_2[E]_0)}{b} =$$
$$(K_S/k_2k_3[E]_0) + ([S]_0/k_2k_3[E]_0) \qquad (9)$$

The left-hand side of eq 9 contains the quantities $[S]_0/k_{cat}[E]_0$ and $K_s/k_2[E]_0$ which are derived from the intercept and slope, respectively, of the Lineweaver-Burk plot of the steady-state rate data.[19] If the data of Table III are plotted according to eq 9 one obtains $3.0 \times 10^{-4}\ M$ for the value of K_S and $7.7 \times 10^{-2}\ \text{sec}^{-1}$ for k_2. The results of this analysis are fairly self-consistent as far as they go. K_m(app) is calculated from eq 10.[18] Using the microscopic constants on the

$$K_m(\text{app}) = \frac{k_3}{k_2 + k_3} K_s \qquad (10)$$

right-hand side of the equation, K_m(app) is 1×10^{-5} while that obtained from the Lineweaver-Burk plot of the steady state is $1.45 \times 10^{-5}\ M$. In addition the second-order rate constants for the presteady-state and steady-state reactions are 2.6 and $2.0 \times 10^{-2}\ M^{-1}\ \text{sec}^{-1}$, respectively. Table IV expresses the fact that the values for the microscopic constants k_2 and K_S reported above satisfactorily explain the variation observed in the first-order rate constant b with variation in the substrate concentration on the basis of eq 8 derived from mechanism 7. However, this consistency is largely meaningless since the actual values of b have been used to plot eq 9 to evaluate k_2 and K_S.

Table IV. A Correlation of the First-Order Rate Constant b Observed in the Presteady State of the Reaction between Elastase and *p*-Nitrophenyl Trimethylacetate and That Predicted from Microscopic Rate Constants Corresponding to Alternative Mechanisms[a]

$[S]_0 \times 10^5\ M$	Obsd b $\times 10^2\ \text{sec}^{-1}$	b calcd from—Acyl-enzyme with Michaelis–Menten complex[b]	b calcd from—Acyl-enzyme without Michaelis–Menten complex[c]
10.28	2.12	2.22	2.28
8.571	1.87	1.97	1.81
5.811	1.40	1.51	1.56
3.000	0.98	0.96	0.903

[a] Data of Table III. [b] Calculated from $b = \{(k_2 + k_3)[S]_0 + K_s k_3\}/(K_S + [S]_0)$, using $k_2 = 7.7 \times 10^{-2}$, $k_3 = 2.66 \times 10^{-3}$, $K_s = 3.0 \times 10^{-4}$. [c] Calculated from $b = k_{II}[S]_0 + k_3$, according to mechanism of eq 1.

The above treatment ignores the initial rate of the presteady-state reaction. According to the mechanism of eq 7, k_{II}, the second-order rate constant for the initial rate of the presteady-state reaction, should depend on the substrate concentration according to eq 11.[19]

$$k_{II} = \frac{k_2}{K_S + [S]_0} \qquad (11)$$

As is illustrated in Table III, k_{II} is independent of substrate concentration under the conditions employed in the present research. Therefore either the kinetics are inconsistent with mechanism 7 or else $[S]_0$ is much less than K_S.

Mechanism 1, however, is consistent with k_{II}'s being independent of substrate concentration without making any assumptions as to the relative magnitude of any constants. We are therefore obliged to adopt a simple acyl-enzyme mechanism because of the lack of a saturation effect in the presteady state. This is the case despite the extensive precedents in the literature which favor mechanisms involving the Michaelis complex in addition to an acyl-enzyme intermediate.

Actually the adoption of the simpler mechanism not including an adsorptive (Michaelis–Menten) intermediate is not as radical a departure from previous work as it might seem at first glance. It can be regarded as a

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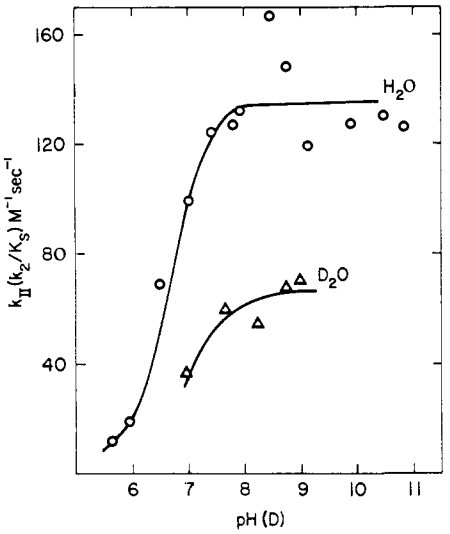

Figure 4. pH- and pD-rate profiles for the presteady-state portion of the reaction between elastase and *p*-nitrophenyl trimethylacetate. Data of Tables V and VII.

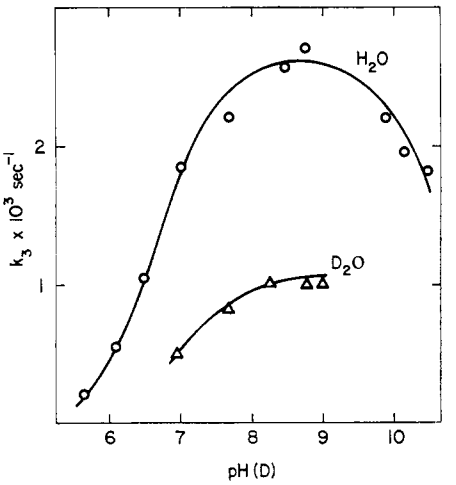

Figure 5. pH- and pD-rate profiles for the steady-state portion o the reaction between elastase and *p*-nitrophenyl trimethylacetate Data of Tables V and VII.

special case of the more general mechanism. Consideration of eq 8 and 11 reveals that the expressions for b and k_{II}, which correspond to mechanism 7, reduce to the form corresponding to the simpler mechanism of eq 1 when $K_S \gg [S]_0$. In these experiments the highest substrate concentration used was at the solubility limit. Thus it is quite reasonable to view the failure to observe a saturation effect in this presteady state as resulting from an unfavorable substrate solubility–K_S relationship rather than from the absence of a Michaelis complex. If we assume that an approach to within 20% of K_S by $[S]_0$ would have resulted in a kinetically observable saturation we may place a lower limit of 5×10^{-4} M on K_S, the dissociation constant of a hypothetical Michaelis complex.[20]

The kinetics of both presteady-state and steady-state reactions were determined throughout the pH range 5.6–10.9. The results are presented in Table V. The quotient k_3/K_m is compared to k_{II} as a check on the self-consistency of the data. Both k_{II} and k_3 depend on a basic functional group whose apparent pK_a is 6.7. In addition, k_3 falls at high pH indicating a dependence on a second group of pK_a between 10.5 and 11.0. The rate of spontaneous hydrolysis becomes unmanageable in this pH range, making it impossible to pinpoint the pH dependency more precisely. The apparent pK_a of the kinetically important functional groups near neutrality was determined by superimposing a semilogarithmic plot of the data over a theoretical log rate–pH curve.[21] The data are presented in the form of conventional pH–rate profiles in Figures 4 and 5.

As in chymotrypsin reactions nucleophiles such as tris(hydroxymethyl)aminomethane and methanol affect k_3. When k_3 is measured in Tris buffer systems at pH's greater than 8 the k_3 increases with increasing concentration of Tris. On the other hand, carbonate and barbital buffer systems gave data which show no indication of a nucleophile reaction. The Tris effect is given in Table VI. It is assumed that the neutral species is the reactive one since the effect increases with increasing pH. Extrapolation of the data of Table VI to zero Tris concentration gives a result which agrees well

Table V. The Effect of pH on the Elastase-Catalyzed Hydrolysis of *p*-Nitrophenyl Trimethylacetate

pH	$k_3^a \times 10^3$	$K_m^b \times 10^5$	k_3/K_m	k_{II}^c	Buffer[d]
5.63	0.19	1.44	13.4	11.8	Phosphate
5.95	...	...	...	19.5	Phosphate
6.11	0.55	1.16	47.5	...	Phosphate
6.50	1.06	1.80	59.0	64.5	Phosphate
7.03	1.82	2.26	80.5	99.2	Phosphate
7.45	...	...	...	124.0	Phosphate
7.61	2.20	1.76	125.0	...	Phosphate
7.82	...	...	...	127.0	Phosphate
7.96	...	...	...	132.0	Phosphate
8.49	2.56	1.58	162.0	167.0	Barbital
8.79	2.51	1.60	157.0	148.0	Barbital
9.15	...	...	...	119.0	Carbonate
9.92	2.20	1.57	140.0	127.0	Carbonate
10.18	1.93	1.44	134.0	...	Carbonate
10.50	1.83	1.39	131.0	130.0	Carbonate
10.87	...	...	...	126.0	Carbonate

[a] From steady-state reaction; $k_3 = V_{max}/[E]_0$. [b] From Lineweaver–Burk plot of steady state. [c] From presteady state; k_{II} = initial rate/$[E]_0[S]_0$. [d] $I = 0.05$.

Table VI. The Effect of Tris(hydroxymethyl)aminomethane and Methanol on the Kinetics of the Elastase-Catalyzed Hydrolysis of *p*-Nitrophenyl Trimethylacetate[a]

I	Tris,[b] M	$k_3 \times 10^3$	Methanol, mole fraction	V_{max},[c] M sec^{-1} $\times 10^8$
0.005	0.00	2.6	0.00	0.47
0.05	0.36	2.84	0.08	1.1
0.05	0.144	3.28	0.037	0.95
0.30[d]	0.267	4.10	0.057	0.80
0.30[d]	0.534	4.86	0.078	4.0
0.05	0.057	2.62	0.123	2.5
0.05	0.144	3.18		
0.05	0.364	3.90		

[a] pH 7.9, 25°, 3.1% (v/v) acetonitrile–water. [b] Concentration of the neutral species. [c] Tris, $I = 0.05$, pH 8.57; 25°, 1.6% (v/v) acetonitrile–water; $[E]_0 = 1.82 \times 10^{-6}$ M. [d] KCl added.

(20) *Cf.* L. Faller and J. M. Sturtevant, *J. Biol. Chem.*, **241**, 4825 (1966).

(21) L. J. Brubacher and F. J. Kézdy, unpublished manuscript.

with the k_3 measured in barbital and carbonate buffer systems. The magnitude of the Tris effect is considerably less than that seen in chymotrypsin reactions. The effect of methanol on k_3, or rather V_{max}, is also given in Table VI. The behavior is not simple. Two maxima occur with increasing methanol concentration. The methanol effect on the catalytic rate constant is recorded in terms of V_{max} rather than k_3 in recognition of the fact that since the enzyme has not been titrated in methanolic medium we can only assume that $[E]_0$ is constant throughout the experiments of Table VI.

The elastase-catalyzed hydrolysis of *p*-nitrophenyl trimethylacetate was also carried out in deuterium oxide. The kinetic results paralled those of the water reaction and are internally consistent, indicating that the gross mechanism, insofar as it is reflected in the kinetics, probably does not change on going from water to deuterium oxide. The effect of pD on the kinetic constants is presented in Figures 4 and 5. Theoretical pD–rate profiles, depending on basic groups of pK = 7.05 and 6.92 for the presteady and steady state, respectively, are found. The data appeared to be well behaved in the sense that they are consistent with the dependence on a single ionizing group. The deuterium oxide solvent isotope effects are: (1) a decrease of the plateau-limiting velocity constants; and (2) an increase in the pK of the kinetically important ionizations. These kinetic and equilibrium effects are summarized in Table VII.

Table VII. Deuterium Oxide Kinetic and Equilibrium Effects

	H_2O	D_2O	k_H/k_D
k_3	2.58 ± 0.03	1.06 ± 0.03	2.4 ± 0.1
k_{II}	150 ± 10	67 ± 5	2.0 ± 0.3
pK_a[a]	6.70 ± 0.04	7.05 ± 0.1	...
pK_a[b]	6.75 ± 0.05	6.92 ± 0.15	...

[a] pK_a of the basic group seen in k_3. [b] pK_a of the basic group seen in $k_{II}(k_3/K_m)$.

Kinetic analysis of the elastase-catalyzed hydrolysis of *o*-nitrophenyl trimethylacetate parallels that of the *para* isomer as is shown in Table VIII. It is apparent

Table VIII. Kinetics of the Reaction between Elastase and *o*-Nitrophenyl Trimethylacetate[a]

$[S]_0 \times 10^4$ M	$b \times 10^3$ sec^{-1}	k_{II},[b] M^{-1} sec^{-1}	$k_{II}[E]_0 \times 10^3$ sec^{-1}	$(k_{II}[S]_0 + k_3) \times 10^3$ sec^{-1}	$V \times 10^9$ [c] M sec^{-1}
2.14	3.8 ± 0.5	10.4	2.2	3.8	5.77
1.14	4.6 ± 1.0	13.7	1.6	3.2	5.00
0.565	2.9 ± 1.0	15.1	0.85	2.4	3.20

[a] 25.0°, pH 7.56 phosphate, I = 0.05, 1.6% (v/v) acetonitrile–water, $[E]_0 = 5.1 \times 10^{-6}$ M, lot 6404. [b] k_{II} = initial rate/$[E]_0 \cdot [S]_0$. [c] Steady-state turnover rate.

that the precision of these experiments, especially the presteady state, is less than that of the *para* isomer. The results do, however, support the assumption that the *ortho* isomer reacts *via* the same mechanism as the *para* isomer. The most striking feature of the results which are summarized in Table IX are the following:

Table IX. A Comparison of the Limiting Kinetic Constants for *o*- and *p*-Nitrophenyl Trimethylacetate

	$k_3 \times 10^3$ sec^{-1}	k_{II}, M^{-1} sec^{-1}
o-Nitrophenyl trimethylacetate	2.25	16.8
o-Nitrophenyl trimethylacetate	2.52[a]	...
p-Nitrophenyl trimethylacetate	2.60	150

[a] A result of a set of four experiments done under conditions which expedite measuring of k_3.

(1) k_{II}, the second-order rate constant for the presteady state, is 7.5 times lower for the *ortho* isomer than for the *para* isomer; and (2) the maximum turnover velocity constant is essentially the same for the two reactions within experimental error. Bearing in mind the fact that the basic saponification rate of the *ortho* isomer is 4.9 times slower than the *para*, we see that the relative acylation rates are well correlated by the saponification rates. More importantly, we observe strong support for the acyl-enzyme hypothesis since the similarity in k_3 can be best explained by invoking the rate-determining reaction of a common intermediate in the enzymatic process. The common intermediate can reasonably be designated as trimethylacetyl-elastase.

Discussion

The kinetic analysis utilized here is predicated on the occurrence of an acyl-enzyme intermediate. It is difficult to explain the kinetic data in any other simple manner. The occurrence of an acyl-enzyme intermediate is not surprising by analogy with the action of other proteolytic enzymes. The evidence for the formation of an acyl-enzyme intermediate includes: (1) the biphasic rate of appearance of *p*-nitrophenol including the effects of enzyme and substrate concentration on each portion of the reaction; (2) the identity of k_3 for the hydrolysis of *o*- and *p*-nitrophenyl trimethylacetates whereas the hydroxide ion rate constants are 4.9-fold different; and (3) the accelerating effect of tris(hydroxymethyl)aminomethane and methanol on the turnover rate of the enzymatic reaction.

What functional groups are involved in the catalysis? Presumably the site of acylation is the same hydroxymethyl group of serine which is uniquely phosphorylated by diisopropyl phosphorylfluoridate.[6] Chemically, there is every reason to expect diisopropyl phosphorylfluoridate, diethyl *p*-nitrophenyl phosphate,[6] and carboxylic esters to react in an analogous fashion. The stoichiometric nature of the phosphorylation and acylation reactions examined in this and previous research confirm this expectation.

The pH dependencies of the steady-state and presteady-state portions of the reaction are quite similar to those of trypsin and chymotrypsin.[22,23] The steady-state (k_3) reaction shows a bell-shaped dependency with pK_a's of 6.7 and 10.5–11.0. The presteady state (k_{II}) shows a sigmoid dependency based on a pK_a of 6.7. The two dependencies on pK_a's of 6.7 are indicative of the operation of an imidazole moiety of the histidine residue in both acylation and deacylation. Two histidine residues have been shown to be present in the amino

(22) M. L. Bender, J. V. Killheffer, Jr., and F. J. Kézdy, *J. Am. Chem. Soc.*, **86**, 5330 (1964).
(23) M. L. Bender, M. J. Gibian, and D. J. Whelan, *Proc. Natl. Acad. Sci. U. S.*, **56**, 833 (1966).

acid sequence of elastase in relative positions which are identical with those found in trypsin and chymotrypsin but as yet a histidine has not been directly shown to be involved in the enzymic process through photooxidation, alkylation, or other modification studies. It is more difficult to identify the acid whose pK_a is 10.5–11.0. The X-ray crystallographic structure of chymotrypsin has recently revealed an ionic bond between the carboxylate side chain of aspartic acid residue 194 and the protonated α-amino group of an N-terminal isoleucine as part of its active site.[24] Elastase contains this same aspartic acid residue in the primary structure of its active site. In view of the well-known structural and mechanistic similarities reported here between elastase and chymotrypsin it is possible to tentatively identify the acid group of pK_a = 10.5–11.0 as an α-amino group of isoleucine.

If we accept the catalytic participation of imidazole we are faced with the problem of assigning its catalytic function to that of a nucleophile or of a general base.

The large deuterium oxide effect found in the present research parallels similar large effects in chymotrypsin- and trypsin-catalyzed reactions.[25,26] These deuterium oxide solvent isotope effects coupled with the observation of the reaction of the acyl-enzymes with nucleophiles dependent on the first power of the nucleophile concentration lead to specification of the catalytic function of the imidazole as a general base rather than nucleophilic catalyst.[27] In elastase-catalyzed reactions both deuterium oxide isotope effects and nucleophilic reactions of the acyl-enzyme are seen. Therefore the same general conclusion may be made. It should be noted that using deuterium oxide solvent isotope effects as sole criterion for distinguishing between nucleophilic and general base catalysis is subject to considerable uncertainty.[28]

The mechanistic scheme used to analyze the kinetics of elastase catalysis does not contain a Michaelis complex usually seen in enzyme reactions. This formulation then implies that acylation is a bimolecular process. It is difficult, however, to imagine such an enzymatic reaction since intramolecular reactions are considerably more facile than the corresponding intermolecular processes. Therefore the lack of saturation observed in the elastase system is more probably due to an unfavorable K_S, considerably larger than the solubility of the substrate employed.

In conclusion, this research indicates that the mechanism of elastase-catalyzed ester hydrolysis bears a striking similarity to that of other serine proteinases such as chymotrypsin and trypsin.

Appendix

Derivation of Kinetic Equations. The numbers to the right of an expression correspond to its number in the text of this manuscript.

$$E + S \underset{}{\overset{k_{II}}{\longrightarrow}} ES \overset{k_3}{\longrightarrow} E + P_2 \qquad (1)$$
$$+ P_1$$

We define

$$d[P_1]/dt = k_{II}[E][S]_0$$
$$d[ES]/dt = k_{II}[E][S]_0 - k_3[ES]$$

and assume $[S]_0$ is always much greater than $[E]_0$.

Steady-State Reaction. We assume a steady state in [ES]

$$d[ES]/dt = k_{II}[E][S]_0 - k_3[ES] = 0$$
$$k_{II}[E][S]_0 = k_3[ES] \qquad [E]_0 = [ES] + [E]$$
$$[E](k_{II}[S]_0 + k_3) = k_3[E]_0 \qquad v = k_{II}[E][S]_0$$
$$v = \frac{k_{II}k_3[E]_0[S]_0}{k_{II}[S]_0 + k_3} = \frac{k_3[E]_0[S]_0}{[S]_0 + k_3/k_{II}} \qquad (4)$$

Presteady-State Reaction.

$$\frac{d[ES]}{dt} = k_{II}[E][S]_0 - k_3[ES] \qquad [E]_0 = [E] + [ES]$$
$$= k_{II}[E]_0[S]_0 - [ES](k_{II}[S]_0 + k_3)$$
$$= a - [ES]b$$

We define $a = k_{II}[E]_0[S]_0$ and $b = k_{II}[S]_0 + k_3$ (eq 2)

$$\frac{d[ES]}{dt} = a - b[ES] \qquad \frac{d[ES]}{a - b[ES]} = dt$$
$$\int_0^{ES} \frac{d(ES)}{a - b[ES]} = \int_0^t dt \qquad [ES] = (a/b)(1 - e^{-bt})$$

Thus b is the first-order rate constant for the attainment of a constant concentration of acyl-enzyme intermediate.

The Relationship between the Burst and the Enzyme Concentration $[E]_0$.

$$\frac{d[P_1]}{dt} = k_{II}[E][S]_0 \qquad [E]_0 = [E] + [ES]$$
$$= k_{II}[E]_0[S]_0 - k_{II}[ES][S]_0$$

recalling that $[ES] = (a/b)(1 - e^{-bt})$

$$\frac{d[P_1]}{dt} = k_{II}[E]_0[S]_0 - k_{II}(a/b)(1 - e^{-bt})[S]_0$$
$$\int_0^{P_1} d[P_1] = \int_0^t (k_{II}[E]_0[S]_0 - k_{II}(a/b)(1 - e^{-bt})[S]_0)dt$$
$$[P_1] = (k_{II}[E]_0[S]_0 - k_{II}(a/b)[S]_0)t - k_{II}(a/b^2)[S]_0(e^{-bt} - 1)$$

at large values of t; $[P_1] = At + \pi$ where

$$A = k_{II}[S]_0\{[E]_0 - (a/b)\}$$

and

$$\pi = \frac{(k_{II}[S]_0)^2[E]_0}{(k_{II}[S]_0 + k_3)^2} \qquad (5)$$

rearranging (5) we obtain

$$(\pi/[E]_0)^{1/2} = \frac{k_{II}[S]_0}{k_{II}[S]_0 + k_3} = \frac{1}{1 + k_3/(b - k_3)}$$

and

$$1/[\pi]^{1/2} = 1/[E]_0^{1/2} + k_3/(k_{II}[S]_0[E]_0^{1/2}) \qquad (6)$$

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(25) M. L. Bender and G. A. Hamilton, *J. Am. Chem. Soc.*, **84**, 2570 (1962).
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(27) M. L. Bender and F. J. Kézdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964).
(28) J. K. Stoops, Ph.D. Thesis, Northwestern University, 1966.