Mechanism of Chymotrypsin: Concurrent Chemical and Enzymic Aminolysis of an Acyl-enzyme of Methylchymotrypsin. Efficiency of Acid–Base Catalysis

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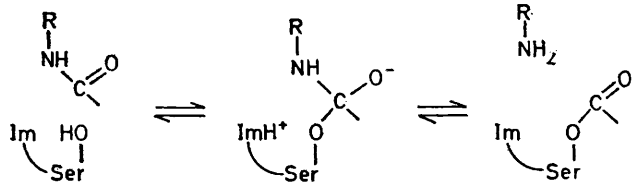
An acyl-enzyme of a modified chymotrypsin in which the nitrogen $N^{\epsilon 2}$ of histidine-57 is methylated has been prepared. The rates of deacylation of this acyl-enzyme in the presence of various amines of pK_a between 3.8 and 11 have been measured at several pH values. For hydrazine, two concurrent pathways of aminolysis are observed, one whose rate is proportional to the hydroxide ion concentration and one whose rate is proportional to the fraction of the active site in the non-protonated form with a pK_a close to 7. For the other amines, the rates of aminolysis have been measured above pH 8 and the occurrence of two pathways is detected in most cases. From the slope of the Brønsted plot and the absence of terms second order in amine, the rate-limiting step of the hydroxide ion catalysed pathway is described as serine anion expulsion from the anionic tetrahedral intermediate (T^-). The rate-limiting step of the enzymic pathway is the serine expulsion from T^- catalysed by the conjugate acid of the imidazolyl group of the active site. The efficiency of the acid catalysis of the decomposition of the anionic tetrahedral intermediate is estimated for the modified and the native enzyme. With the native enzyme, this contribution to the overall catalysis is very high. Attempts to observe large rate enhancements due to intramolecular catalysis in the aminolysis by diamines were unsuccessful. When the active site is modified, the leaving group specificity observed with the native enzyme and amino-acid amides is lost.

A CONSIDERABLE amount of work has been devoted in recent years to the investigation of the mechanism of hydrolysis of amides and peptide bonds by chymotrypsin and related enzymes. The main features of the mechanism have been reviewed.[1,2]

The enzymic hydrolysis process is a multistep reaction. After the Michaelis complex, an acyl-enzyme is formed in a slow step from which the free enzyme is regenerated quickly by the hydrolysis of the ester function.[3,4] In the formation of the acyl-enzyme, a tetrahedral intermediate has been postulated by analogy with the corresponding reaction in solution (alcoholysis of amides or the reverse reaction aminolysis of esters).[5] Recently, the accumulation of a tetrahedral intermediate has been detected in the hydrolysis of anilides bearing strong electron-withdrawing groups catalysed by enzymes analogous to chymotrypsin: α-lytic protease, elastase, and trypsin.[6,7]

The rate of the reaction is known to be dependent on the protonation state of histidine-57. The imidazolyl side chain acts as an acid–base catalyst by removing the proton from serine-195 and protonating the leaving amine (Scheme 1).



SCHEME 1

A few questions remain unsolved regarding the mechanism of these enzymes. (1) Except in the special cases where the tetrahedral intermediate accumulates, the available data on the hydrolysis of amides or the aminolysis of acyl-enzymes [8–12] do not allow a definite attribution of the rate-limiting step, *i.e.* whether it entails formation or decomposition of the tetrahedral intermediate; in fact, the β value of the Brønsted plot is intermediate between those found for rate-determining attack and rate-determining breakdown of tetrahedral intermediates.[9,13] (2) Histidine-57 acts as an intramolecular general acid–base catalyst. What is the efficiency of that catalysis compared with intermolecuar catalysis and what is the detailed mechanism of proton transfer?

In an effort to provide additional insight into these matters, we have studied the kinetics of aminolysis of an acyl-enzyme of a chymotrypsin molecule modified by methylation of $N^{\epsilon 2}$ according to the method of Nakagawa and Bender.[14] This modified enzyme is known to have a low residual inherent activity.[15] Furthermore, it has been shown that the hydrolysis of the acyl-enzyme of methylchymotrypsin can be catalysed by hydroxide ions in a non-enzymic reaction within the solvation framework of the active site.[16] Hopefully, an analogous chemical pathway could be observed for the aminolysis, helping to close the gap between the best chemical models [17,18] and the true enzymic reactions. Finally, the system seems to be suitable to test the stereochemical model proposed earlier [19] to explain the enhanced reactivity of amino-acid amides in the aminolysis of acyl-enzymes of specific substrates.

EXPERIMENTAL

Materials.—Three times crystallized dialysed salt-free and lyophilized α-chymotrypsin was obtained from Sigma (lots 92C-8120 and 51C-8050). Methylchymotrypsin was prepared and purified as reported previoudly.[16] Proflavin hemisulphate was purchased from Sigma and recrystallized from methanol–water in the dark. Propylamine, ethanolamine, β-alanine, and hydroxylamine hydrochloride (puriss. p.a. Fluka), hydrazine hydrate (AnalaR B.D.H.), Tris

(puriss. Koch-Light), and ammonia (Baker Analyzed Reagents) were used without further purification. The other commercially available amines (semicarbazide hydrochloride, glycine, methoxyethylamine, ethylenediamine hydrate, and propylenediamine) were purified by crystallization or distillation. Glycinamide and alaninamide hydrochlorides were prepared as described previously.[3] Trifluoroethylamine was prepared by reduction of trifluoroacetamide with $LiAlH_4$ according to the method of Bissel and Finger [20] and its hydrochloride was recrystallized from propan-2-ol. *N*-Acetyl-l-phenylalanine *p*-nitrophenyl ester was synthesized according to Ingles and Knowles,[21] m.p. 139—140 °C (lit., 140 °C).

Methods.—The modified enzyme titration and the deacylation of *N*-acetyl-l-phenylalanyl(methyl)chymotrypsin were run as described previously,[16] except that the deacylation was initiated by addition of 0.5 ml of stock amine solution or a mixture of stock amine solution and 1M-KCl. For the low pK_a amines a mixture of amine and borate or mostly carbonate buffer was used to improve the buffering capacity. The final pH values of the solutions were checked at the end of the kinetic runs. Experiments for which the pH deviated by 0.03 or more from the mean of the series were discarded. The ionic strength was maintained at 1.0 either by the conjugate acid of the amine, KCl, or the buffer. For basic amines, the percentage of free amine in the stock solution was measured by titration with 1N-HCl when it was <10%.

The rates of deacylation of acetyl-l-phenylalanylchymotrypsin were determined as described previously [3] on a Durrum stopped-flow spectrometer.

The values of the pseudo-first-order rate constants were obtained from linear plots of log $(A_t - A_\infty)$ against time. Second-order rate constants k_{am} were determined from plots of k_{obs} *versus* amine concentration. The k_{am} rate constants obtained above pH 8.4 were then plotted against the hydroxide ion concentration to get the k_{am° and the $k_{am.OH}$ terms (see Results section). For hydrazine, measurements were done over a larger pH range; the individual rate constants of deacylation were used directly to obtain the parameters of equation (1). The values of k_0, k_{OH}, k_{am°, $k_{am.OH}$, and K_a were varied to minimize the sum of the squares of the

$$k_{obs} = \frac{k_0 K_a}{K_a + [H^+]} + k_{OH}[OH] + \frac{k_{am^\circ} K_a}{K_a + [H^+]} + k_{am.OH}[OH] \quad (1)$$

relative residuals $(k_{obs} - k_{calc})/k_{calc}$. The following values were obtained: $k_0 = (2.50 \pm 0.07) \times 10^{-3}$ s^{-1}; $k_{OH} = (6.8 \pm 0.2) \times 10^1$ l mol^{-1} s^{-1}; $k_{NH_2NH_2^\circ} = (1.28 \pm 0.04) \times 10^{-3}$ l mol^{-1} s^{-1}; $k_{NH_2NH_2.OH} = (8.6 \pm 0.1) \times 10^3$ l mol^{-1} s^{-1}; and $pK_a = (6.85 \pm 0.03)$.

RESULTS

With most amines, the observed rate constants of deacylation of the acyl-enzyme (k_{obs}) depended linearly on the free amine concentration but the slope and the intercept were pH dependent as shown for hydroxylamine in Figure 1. The intercept reflects the hydrolysis reaction described in a previous paper,[16] the slope, the aminolysis reaction [equation (2)]. With ammonia, the rate of deacylation has

$$k_{obs} = k_{hydr} + k_{am}[Am] \quad (2)$$

been measured as a function of the concentration up to a value of 2M in free amine at pH 9.38 with an ionic strength

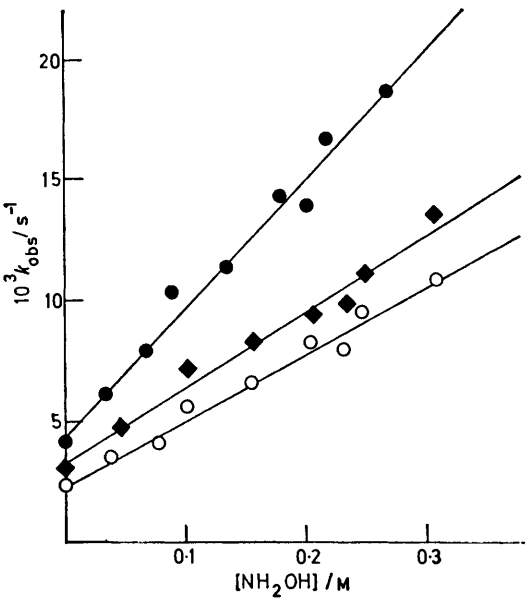


FIGURE 1 Observed first-order rate constants of deacylation of *N*-acetyl-l-phenylalanyl(methyl)chymotrypsin as a function of the hydroxylamine concentration at 25 °C and ionic strength 1.0: ● pH 9.38; ♦ pH 8.87; ○ pH 8.44

of 2. Even with this non-hindered amine no upward deviation from linearity arising from a term second order in amine could be detected.

For hydrazine, the rates of aminolysis have been determined at several pH values. The data are collected in

$$k_{NH_2NH_2} = \frac{k_{NH_2NH_2^\circ} K_a}{[H^+] + K_a} + k_{NH_2NH_2.OH}[OH] \quad (3)$$

Figure 2. The second-order rate constants can be described by equation (3) with a pK_a of 6.85 close to that found in the

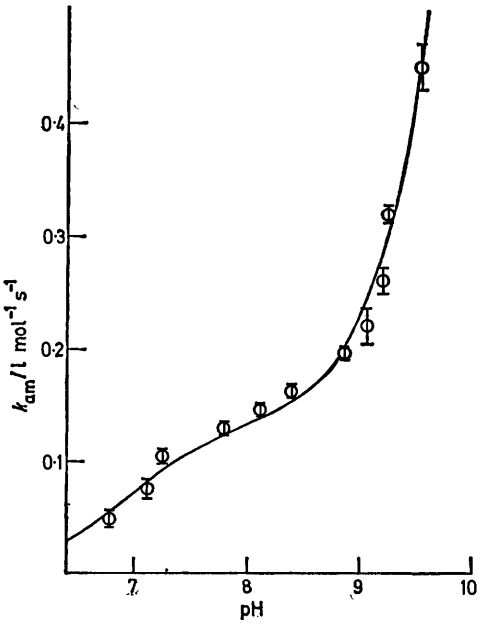


FIGURE 2 pH Profile of the rate constant of hydrazinolysis of *N*-acetyl-l-phenylalanyl(methyl)chymotrypsin at 25 °C and ionic strength 1.0

study of the hydrolysis reaction alone.[16] At pH 7 or below, the rate of aminolysis becomes less easy to measure accurately because the contribution of the aminolysis to the total rate of deacylation is relatively small.

For other amines, the rates of deacylation have been determined at several pH values above 8.4. Plots of the second-order rate constants of aminolysis as a function of pH 9.49; the total rate of deacylation in the presence of 0.17M-hydroxylamine varies from 12.0 ± 0.3 to 14.3 ± 0.6 s^{-1} between concentration limits of the buffer of 0.018 and 0.11M with some deviation from linear first-order plots at high buffer concentration. Because these variations are not much larger than the standard deviation on the individual measurements no systematic study of the effects of

TABLE 1

Enzyme catalysed and hydroxide ion catalysed rate constants for the aminolysis of *N*-acetyl-1-phenylalanyl(methyl)-chymotrypsin at 25 °C, ionic strength 1.0

No.	Amine	pK_a	k_{am^0}/l mol^{-1} s^{-1}	$k_{am.OH}$/l^2 mol^{-2} s^{-1}
1	$NH_2CONHNH_2$	3.86 [a]	(2.65 ± 0.08) 10^{-3}	(3.88 ± 0.03) 10^2
2	$CF_3CH_2NH_2$	5.84 [b]	1 × 10^{-3}	(7.0 ± 3.0) 10^3 [e]
3	NH_2OH	6.10 [b]	(2.24 ± 0.08) 10^{-2}	(1.27 ± 0.06) 10^3
4	NH_2NH_2	8.20 [d]	(1.28 ± 0.04) 10^{-1}	(8.7 ± 0.1) 10^3
5	$NH_2CH_2CONH_2$	8.22 [a]	(5.75 ± 0.18) 10^{-2}	(2.4 ± 0.2) 10^3
6	NH_3	9.40 [c]	(6.55 ± 0.50) 10^{-1} [f]	8 × 10^2 [g]
7	$CH_3OCH_2CH_2NH_2$	9.72 [c]	(2.20 ± 0.10) 10^{-1} [f]	3 × 10^2 [g]
8	$CH_3CH_2CH_2NH_2$	10.89 [d]	(8.5 ± 0.2) 10^{-1}	(5.45 ± 0.43) 10^3

[a] Ref. 3. [b] Ref. 30. [c] Determined from measurements of the pH of partially neutralized solutions. [d] Ref. 19. [e] Plots of k_{obs} *versus* amine concentration are not linear (see text). [f] Mean of measurements at 4 pH values. [g] Maximum value determined as described in the text.

the hydroxide ion concentration (Figure 3) allow a separation of k_{am^0} as the intercept and $k_{am.OH}$ as the slope according to equation (4).

$$k_{am} = k_{am^0} + k_{am.OH}[OH] \quad (4)$$

For the amines of low pK_a, it was necessary to add a buffer to maintain the pH; carbonate or borate buffers

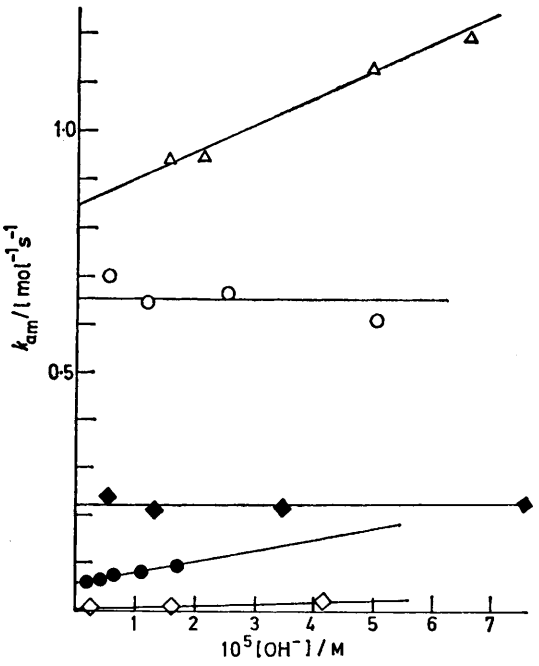


FIGURE 3 Second-order rate constants of aminolysis of *N*-acetyl-1-phenylalanyl(methyl)chymotrypsin as a function of the hydroxide ion concentration at 25 °C and ionic strength 1.0: △ propylamine; ○ ammonia; ♦ methoxyethylamine; ● glycinamide; ◇ semicarbazide

were used. It was checked that the buffer did not affect the rate of deacylation significantly. The rate of hydrolysis of the acyl-enzyme varies from 3.8 to 4.1 × 10^{-3} s^{-1} when the total buffer concentration changes from 0.025 to 0.1M at buffers was undertaken. For the measurements of the rates of aminolysis, the total buffer concentration was always kept below 0.05M and in most cases below 0.025M. Tris buffers

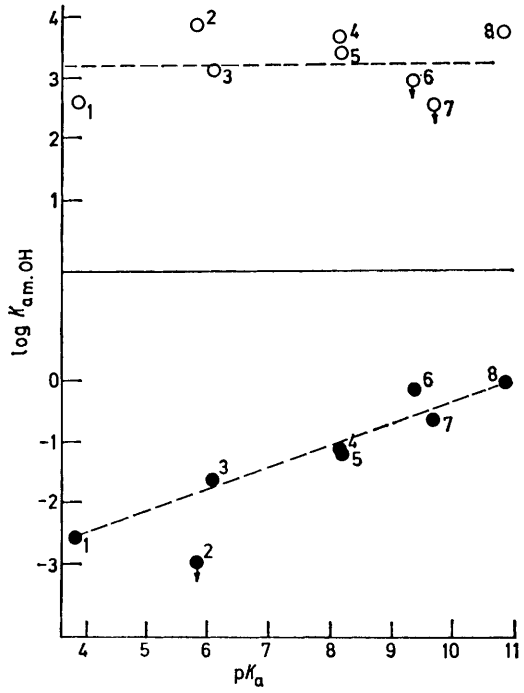


FIGURE 4 Brønsted plots for the dependence of the enzyme catalysed k_{am^0} (●) and the hydroxide ion catalysed $k_{am.OH}$ (○) rates of aminolysis of *N*-acetyl-1-phenylalanyl(methyl)-chymotrypsin on the pK_a of the amines; numbers refer to items in Table 1

were avoided because Tris catalyses the deacylation significantly. For the amines of low pK_a, the contribution of the aminolysis to the total rate of deacylation becomes low around pH 8 so that k_{am^0} is difficult to measure with high accuracy.

The rate constants are collected in Table 1 and used to construct the Brønsted plots (Figure 4) in which the rate

constants of hydrazine have been statistically corrected. For ammonia and methoxyethylamine, no increase in second-order rate constants was observed as a function of the pH; a maximum third-order rate constant $k_{am.OH}$ was calculated by assuming a 10% error on the k_{am} at the lowest and highest hydroxide ion concentration (over- and under-estimation respectively). For trifluoroethylamine, only approximate rate constants can be given because the aminolysis pathway contributes very little to the deacylation below pH 8.5 and above 9, the plots of rates of deacylation *versus* amine concentration showing a strong curvature as observed for a saturation phenomenon. The second-order rate constants k_{am} were obtained as the intercept from plots of $(k_{obs} - k_{hydrol})/[Am]$ as a function of $(k_{obs} - k_{hydrol})$ (inversed Eadie plots); the apparent binding constants are of the order of 2×10^{-2}M. Presumably, there is formation of a complex between the enzyme ' leaving group site ' and the amine. Why is trifluoroethylamine the only amine to show this behaviour is not clear. It might be that binding is not detected with the other relatively hydrophobic amines (propylamine and eventually methoxyethylamine) because being more reactive they are engaged at lower concentration as free amine.

A methylchymotrypsin-catalysed aminolysis of an ester has been studied by Byers and Koshland,[22] the hydroxylaminolysis of *N*-acetyl-l-tyrosine ethyl ester. They report a rate constant of 4.65×10^{-2} l mol^{-1} s^{-1} at pH 7.8. From our data, we calculate a rate constant of $2,3 \times 10^{-2}$ l mol^{-1} s^{-1} for the hydroxylaminolysis of *N*-acetyl-l-phenylalanyl-(methyl)chymotrypsin at the same pH. The hydrolysis of tyrosine derivatives by chymotrypsin is always somewhat faster than the hydrolysis of phenylalanine derivatives as shown by the ratio of the rates of deacylation of *N*-acetyl-l-tyrosyl- and *N*-acetyl-l-phenylalanyl-chymotrypsin, 1.4,[23] or the ratio of the $k_{max.}$ values for the hydrolysis of *N*-acetyl-l-tyrosinamide and *N*-acetyl-l-phenylalaninamide, 3.[24]

The aminolysis by diamines has been investigated with ethylenediamine and propylenediamine. An extension of the equation for monoamines above pH 8 leads to equation (5) for the observed rate of aminolysis where the constants

$$k_{am} = k_{DA}[DA] + k_{DA.OH}[DA][OH] + k_{DAH^+}[DAH^+] + k_{DAH^+.OH}[DAH^+][OH] \quad (5)$$

k_{DA} and $k_{DA.OH}$ represent the deacylation by the free amine and k_{DAH^+} and $k_{DAH^+.OH}$ by the monocation. This equation can be rearranged to express the aminolysis rate constant as a function of the pH and the analytical diamine concentration [equation (6) where $K_1 = 10^{-7.46}$ and $K_2 =$

$$\frac{k_{am}}{DA^0}\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right) = k_{DAH^+} + \left(k_{DA}\frac{K_2}{K_w} + k_{DAH^+.OH}\right)[OH^-] + k_{DA.OH}\frac{K_2}{K_w}[OH^-]^2 \quad (6)$$

$10^{-10.16}$ are the acidity constants of the dication and the monocation, respectively]. Plots of the left side of the equation against the hydroxide ion concentration should give a straight line or a curve depending on the presence of an hydroxide-ion catalysed term for the free amine. Such a plot is shown for the deacylation by ethylenediamine between pH 8.12 and 9.63 in Figure 5, leading to the determination of $(k_{EDA}K_2/K_w + k_{EDAH^+.OH}) = (5.3 \pm 0.6) \times 10^4$ l^2 mol^{-2} s^{-1}; no upward curvature corresponding to an hydroxide ion-catalysed aminolysis by the free diamine is seen. A determination based on two pH values for propylenediamine leads to the measured value of 2.7×10^4 l^2 mol^{-2} s^{-1}.

The rates of aminolysis have been measured with a few more amines at a single pH to check whether the high

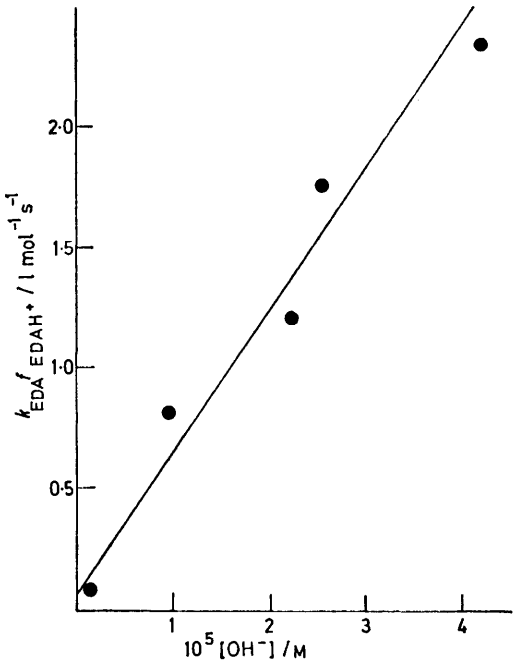


FIGURE 5 Aminolysis of *N*-acetyl-l-phenylalanyl(methyl)-chymotrypsin by ethylenediamine: second-order rate constants between pH 8.12 and 9.63 times the fraction of monocation as a function of the hydroxide ion concentration

reactivity was peculiar to diamines, and see whether the reactivity order $k_{alaninamide} > k_{glycinamide} > k_{hydrazine}$, indicative of leaving group specificity in favour of amino-acid derivatives was maintained with the modified enzyme. The data are collected in Table 2.

We have tried to measure the reactivity of ethylenediamine toward the corresponding acyl-enzyme of native

TABLE 2

Second-order rate constants of aminolysis of *N*-acetyl-l-phenylalanyl(methyl)chymotrypsin at 25 °C, ionic strength 1.0, and fixed pH

Amine	pK_a	pH	k_{am}/l mol^{-1} s^{-1}
$NH_2CH_2CONH_2$	8.22 [a]	9.05	0.082 ± 0.005
L-$NH_2CH(CH_3)CONH_2$	8.24 [a]	8.90	0.061 ± 0.003
NH_2NH_2	8.20 [a]	9.06	0.221 ± 0.016
Tris	8.17 [b]	9.09	0.006 9 ± 0.001 3
$CH_3OCH_2CH_2NH_2$	9.72 [b]	9.54	0.214 ± 0.009
$HOCH_2CH_2NH_2$	9.76 [b]	9.45	0.154 ± 0.026
Glycine	9.76 [b]	9.51	0.241 ± 0.006
β-Alanine	10.15 [b]	9.55	0.160 ± 0.028

[a] Ref. 19. [b] Determined from measurements of the pH of partially neutralized solutions.

chymotrypsin. The observed rate constants of deacylation are described by equation (7). The measurements have

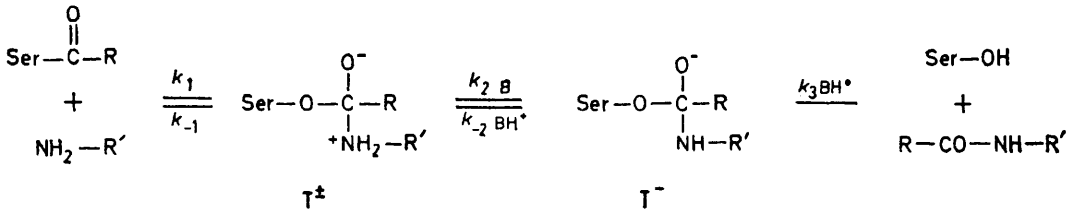
$$k_{obs} = k_{H_2O} + k_{EDA}[EDA] + k_{EDAH^+}[EDAH^+] \quad (7)$$

been done between pH 8.3 and 9.3; unfortunately in this

pH range the first and the third terms are dominant and the technique does not allow measurements at significantly higher pH so that only a very approximate value can be obtained for k_{EDA}: $k_{EDA} = (6 \pm 3) \times 10^2$ l mol^{-1} s^{-1}; $k_{EDAH^+} = (4.3 \pm 0.4) \times 10^2$ l mol^{-1} s^{-1}.

DISCUSSION

The amine-catalysed deacylation of *N*-acetyl-l-phenylalanyl(methyl)chymotrypsin can in principle be interpreted as an amine-catalysed hydrolysis or an aminolysis reaction. The hydroxide ion-catalysed pathway must obviously correspond to an aminolysis. On the other hand, in their investigation of the methylchymotrypsin catalysed hydroxylaminolysis of *N*-acetyl-l-tyrosine ethyl ester, Byers and Koshland observed the formation of a hydroxamic acid. We have tried to measure the amide : acid ratio in the product of hydroxylaminolysis of the acyl-enzyme as a function of the hydroxylamine concentration but the low sensitivity of the hydroxamic acid assay and the difficulty of acylating quantitatively the methylchymotrypsin at the high concentration required for the assay prevented this determination.



SCHEME 2

The rate of the aminolysis reaction of the acyl(methyl)-chymotrypsin is much slower than the rate of the corresponding reaction with the acyl-enzyme of the native chymotrypsin. Because of this, a hydroxide ion-catalysed pathway represents by the $k_{am.OH}[OH]$ term becomes observable besides the enzyme-catalysed pathway represented by the $k_{am}K_a/(K_a + [H^+])$ term. Consequently, the pH dependence of the rate constant of the hydrazinolysis reaction is more complex (Figure 2) than the well known pH profile of the reactions catalysed by the native enzyme where the activity reaches a plateau above pH 7 when the histidine of the active site responsible for the enzymic acid–base catalysis is deprotonated.

If the generally accepted tetrahedral intermediate is explicitely taken into account, the overall reaction is described by Scheme 2 where B and BH^+ stand for the possible catalysing species, the modified histidine of the active site, the hydroxide ion, the amine, the species of the buffer and their respective conjugate acids.

The results of the kinetic analysis can be used to describe more precisely the mechanism of aminolysis. We shall first analyse the data of the hydroxide ion catalysed pathway.

(1) *The Hydroxide Ion-catalysed Pathway.*—For a hydroxide ion-catalysed pathway, the transition state must be anionic. The rate-limiting step can be either the deprotonation of $T^{\pm}$ by a hydroxide ion or the uncatalysed decomposition of T^- to products. Several points favour the second possibility.

(a) The Brønsted plot with a β value close to zero indicates that the nitrogen of the amine is nearly neutral in the transition state. This is consistent with the rate-limiting step being the expulsion of the serine anion from T^-. In their investigation on the aminolysis of methyl formate, Blackburn and Jencks [25] have shown that at some pH value below the pK_a of the amine there is a change in rate-determining step and the alcoholate expulsion becomes limiting; a β value of *ca.* −0.2 can be calculated from their data in that pH range.* The similarity between this value obtained with a system where the rate-limiting step could be unambiguously assigned and the β value of the hydroxide ion-catalysed pathway suggest that here too the rate-determining step is the decomposition of T^- to products. Furthermore, a β value close to zero is not consistent with the rate-limiting step being the deprotonation of $T^{\pm}$. The deprotonation is itself a two-step process, the diffusion of the hydroxide ion toward $T^{\pm}$ and the proton transfer. Because the hydroxide ion is much more basic than the amine in the tetrahedral intermediate, the proton transfer takes place on every encounter and the rate-limiting step is in fact the transport process. At that stage, the nitrogen is still bearing a full positive charge so that a β value close to 1 would be expected if this was the rate-limiting step.

The interpretation of Brønsted plots with enzymic systems is less dependable than with chemical systems because the influence of steric and hydrophobic parameters can be more important and to some extent mask the influence of the basicity on the reactivity.[8,9] Steric and hydrophobic factors are probably responsible for the scatter observed in the plot. We do not think however that a correction for these secondary interactions if it was possible could change the slope (β) from nearly 0 to 1 as would be required if the rate-limiting step was the deprotonation of $T^{\pm}$.

(b) In the pH range where the hydroxide ion-catalysed pathway is observed, no term second order in amine and only a weak and uncertain buffer catalysis could be

* This value is obtained from a plot of the products of the constants $k_1k_4/k_{-1}k_w$ of ref. 25, these being equivalent to the k_{OH} value in this paper.

detected. In the aminolysis of esters, a large term second order in amine and a significant buffer catalysis are observed when the formation of T^- is rate limiting [12,25] but these terms are small when the decomposition of T^- to products is limiting.[25] The same generalization applies to esters of *N*-acetylserinamide and denatured acetylchymotrypsin.[26,27]

(c) After statistical correction, the $k_{am.OH}$ term for hydrazine (an α effect nucleophile) is only 1.9 times larger than the $k_{am.OH}$ term of glycinamide, an amine of similar pK_a. Again in the aminolysis of methyl formate, in the pH range where the formation of T^- is rate limiting, the ratio of reactivity after correction is larger than 100.*

It is interesting to mention that in the pH range where the decomposition of T^- is rate limiting with this system, the rate-limiting step is thought to be the formation of T^- in the aminolysis of esters of *N*-acetylserinamide. The factors responsible for this difference in behaviour are not obvious. The expulsion of the serinate anion without catalysis might be less easy within the active site than in the bulk of the solvent, because of poor solvation of the negative charge. The methylation of the histidine itself can contribute to retard this process; † alternatively, the precise conformation imposed on the tetrahedral intermediate by the enzyme can favour decomposition toward the reagents by stereoelectronic control.[28,29]

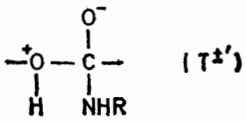
In principle, at some basic pH there should be a change from rate-limiting decomposition to rate-limiting formation of the tetrahedral intermediate T^-; unfortunately, the proflavin displacement technique used here does not allow measurements at sufficiently high pH to check this prediction.

(2) *The Enzymic Pathway.*—The second deacylation pathway is described by the $k_{am}K_a/[H^+] + K_a$ term. The rate-limiting step can be either the transformation of $T^\pm$ into T^- catalysed by the imidazolyl side-chain of the active site or the decomposition of T^- to products catalysed by the conjugate acid of the imidazolyl, in which case the decrease in rate below pH 8 arises because the decrease in the T^- concentration and the increase in the conjugate acid of the imidazolyl concentration as a function of the pH no longer cancel. At pH 8, the formation of T^- is fast compared to its rate of decomposition (see the above discussion on the $k_{am.OH}$ pathway) so that the only way to catalyse the reaction is to increase the rate of transformation of T^- to products by acid catalysis.

This pathway is characterized by a β value around 0.35. The formal positive charge on the nitrogen is significantly larger than in the other pathway. In T^-, the nitrogen is nearly neutral; in the product, it is bearing a formal charge of 0.5.[30] The transition state is thus relatively late with a significant extent of carbon–nitrogen partial double-bond formation and consequently a relatively important carbon–oxygen bond breaking.

* 117 from the ratio of k_1 values, 139 from the ratio of k_2 values.[25]

The question then arises: why is the transition state of the enzymic pathway (acid catalysed C–O bond rupture) later than the transition state of the hydroxide ion-catalysed pathway (uncatalysed C–O bond rupture)? In the uncatalysed pathway, an early transition state is observed as is expected for an exothermic step of a reaction. In the general-acid catalysis of the decomposition of T^-, according to Jencks' rule,[31] the C–O bond rupture and the proton transfer can be concerted because the pK_a of the catalyst is intermediate between the pK_a of the hypothetical oxygen protonated inter-



($T^{\pm\prime}$)

mediate ($T^{\pm\prime}$) and that of the alcohol leaving group (13.6).[32] The catalysed process can be depicted as starting with T^- hydrogen bonded on oxygen with the conjugate acid of the imidazolyl; when the C–O bond begins to break, some proton transfer occurs to facilitate the process but it will become favourable and make a significant contribution to the stabilisation of the transition state only when the pK_a of the oxygen becomes close to 7, in other words when the C–O bond is significantly broken. Early protonation on the oxygen cannot take place because $T^{\pm\prime}$ is too unstable.

The difference in reactivity between the native and the modified enzyme can be at least formally ascribed to the fact that in the native enzyme, there is a much larger proportion of protonated imidazolyl side-chain properly oriented as a hydrogen bond donor to the oxygen of T^-. With the native enzyme obviously, the formation of T^- is also catalysed very efficiently by histidine-57 and no firm conclusion can be reached from the data reported here as to whether the formation or the decomposition of T^- is the rate-limiting step. The Brønsted plot of aminolysis with the native enzyme is quite similar to the one reported here for the enzymic pathway; [19] this suggests that the rate-determining steps are the same. On the other hand, in the cases where a tetrahedral intermediate has been observed to accumulate in the enzymic hydrolysis of *p*-nitroanilides, the breaking of the C–N bond is rate limiting, but the rate difference between the two steps is not large.[6,7] It is in any case possible that different steps are limiting with different amines.

(3) *The Efficiency of the Acid–Base Catalysis.*—The efficiency of the acid catalysis of the decomposition of T^- can be estimated in the following way. With the modified enzyme, going down from the high pH region, the acid catalysis by the conjugate acid of the imidazolyl group appears as a positive deviation from the hydroxide ion-catalysed pathway; this deviation reaches its maximum when the active site is completely protonated, in the pH range where the contribution of the enzymic pathway simplifies to $k_{am}k_a/[H^+]$. The ratio between this value

† We thank a referee for making this suggestion.

and the rate of the hydroxide ion-catalysed pathway at the same pH, $k_{OH}[OH]$, gives the catalytic factor $F_c = k_{am^-} K_a/k_{OH}K_w$. Values of F_c for both the modified and the native enzyme are given in Table 3 for several amines. For comparison, the efficiency of the intermolecular catalysis of decomposition of the anionic tetrahedral intermediate derived from methyl formate and hydrazine by the hydrazinium ion is equal to 1.2 l mol^{-1}.* It should be pointed out that for the native enzyme, if the rate of formation of T^- is limiting, the calculated catalytic factor is underestimated. In any case, it is clear that the contribution of the acid–base catalysis is very high. Although our data do not allow a calculation of the effective molarity of the acid–base catalyst of the active site, they strongly suggest that this value is considerably higher than 10—100M as previously thought [33,34] and falls in the range suggested in a recent study of model compounds to be necessary to account for the observed overall efficiency of enzymic catalysis.[35]

TABLE 3

Calculated catalytic factors for the decomposition of the anionic tetrahedral intermediate derived from the acyl-enzyme and the amine

Amine	$k_{am.OH}K_w$	$k_{MeCt}K_{a\,MeCT}$ [a]	$F_{c\,MeCT}$ [b]	$k_{CT}K_{a\,CT}$ [c]	$F_{c\,CT}$ [b]
$NH_2NHCONH_2$	3.9×10^{-12}	2.9×10^{-10}	75	1.5×10^{-5}	3.8×10^{6}
NH_2OH	1.3×10^{-11}	2.5×10^{-9}	192		
$NH_2CH_2CONH_2$	2.4×10^{-11}	6.3×10^{-9}	262	2.5×10^{-4}	1.0×10^{7}
NH_2NH_2	8.7×10^{-11}	1.5×10^{-8}	172	4.4×10^{-5}	5.1×10^{5}
$NH_2CH_2CH_2CH_3$	5.5×10^{-11}	9.3×10^{-8}	1 690		

[a] Calculated from the k_{am^-} given in Table 1 and $k_{a\,MeCT} = 1.1 \times 10^{-7}$.[16] [b] See text. [c] Calculated from the rate constants of aminolysis of *N*-acetyl-l-phenylalanylchymotrypsin given in ref. 19 and $K_{a\,CT} = 1.5 \times 10^{-7}$ (M. Renard and A. R. Fersht, *Biochemistry*, 1973, **12**, 4713).

With the use of diamines, an attempt has been made to observe a rate enhancement relative to monoamines which would be indicative of intramolecular catalysis. The actual increase in reactivity can be estimated by comparing the observed value of $(k_{EDA}k_2/K_w + k_{EDAH^+.OH})$ with the value expected from the Brønsted plot. For ethylenediamine, the calculated value is $(2 \times 0.6 \times 10^{-10.16}/10^{-14} + 5 \times 10^3) = 1.3 \times 10^4$ l[2] mol^{-2} s^{-1}; the observed value is about four times larger. Propylenediamine is about twice as reactive as expected. The reactivity enhancement observed with these diamines remains small so that the effect is uncertain. For assessment, the data are to be compared with the rate of aminolysis of the native acyl-enzyme; although this rate constant is quite imprecise, the reactivity of ethylenediamine compared to monoamines [19] is also rather high with the native enzyme.

A final interesting observation made in this study is that the rates of aminolysis of the acyl-enzyme of methylchymotrypsin by hydrazine, glycinamide, and alaninamide are quite similar and if anything, hydrazine is more reactive than the other ones. With the native enzyme, amides of amino-acids are much better nucleophiles than hydrazine with reactivity ratios up to 116 after statistical correction. This behaviour was ascribed to a balance of positive and negative interactions between the amino-acid amides and various residues of the active site in which favourable contacts can be formed only at the level of the tetrahedral intermediate and the transition state.[19] The effect of these interactions was observed only with specific acyl-enzymes; they cannot be utilized when the substrate is floppy or held in a wrong orientation. With the modified enzyme, the disruption of the charge-relay system has the same consequences as the change from specific to non-specific substrate.

* $k_{54}k_4$ taken from ref. 25; in the presence of 1M-hydrazinium ion, the rate of the general acid-catalysed decomposition of T^- pathway is nearly equal to that of the uncatalysed pathway.

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