The α -Chymotryptic Hydrolysis of Glycine Esters

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1. The α -chymotrypsin-catalysed hydrolysis of *N*-acetylglycine ethyl and thiolethyl esters was investigated at pH 7.90 and 25° over a wide range of substrate concentrations. 2. The Lineweaver-Burk plots for these substrates are markedly curved, and it is shown that the curvature is due solely to the 'enzyme-blank' reaction. The rate of this reaction is proportional to free enzyme concentration in the range 10–100 μ M, with a pseudo-first-order rate constant of approx. 1×10^{-3} sec.⁻¹. Correction for this reaction by the procedure described leads to linear plots. It is shown that the significance of the enzyme-blank reaction depends on the value of k_0/K_m for the substrate under investigation. 3. Interpretation of the curvature in the Lineweaver-Burk plots by previous workers in terms of activation by excess of substrate is shown to be erroneous. 4. Values of K_m 387 mM and k_0 0.039 sec.⁻¹, and K_m 41 mM and k_0 0.23 sec.⁻¹, were obtained for the ethyl and thiolethyl esters of *N*-acetylglycine respectively. The literature values for the methyl esters of *N*-acetyl- and *N*-propionyl-glycine have been corrected by the procedure described. The new values agree much better with current theories of α -chymotrypsin mechanism and specificity. 5. The kinetic parameters for the ethyl and thiolethyl esters indicate the absence of an electrophilic component in the catalytic mechanism of α -chymotrypsin, and the importance of the ester function in substrate binding.

The Lineweaver-Burk plots for the α -chymotrypsin-catalysed hydrolysis of certain acylated glycine ester substrates are appreciably curved if a wide enough range of substrate concentrations is employed. Wolf & Niemann (1959) and Wolf, Wallace, Peterson & Niemann (1964) have noticed such curvature in their plots for *N*-acetyl- and *N*-propionyl-glycine methyl ester and interpret it in terms of activation by excess of substrate. Substrate activation is considered to arise because the dimensions of the active site of the enzyme are large enough to accommodate more than one molecule of small non-specific substrates such as *N*-acetylglycine methyl ester. At high substrate concentrations, therefore, it is suggested that a ternary ESS complex, in addition to the normal ES complex, is involved. Both complexes can break down to give reaction products. Thus:

They propose that, although the binding of the second substrate molecule is weaker, the substrate orientation for the subsequent catalytic step(s) is improved so that the ternary complex breaks down faster to products than does the binary. The effect of substrate activation on a Lineweaver-Burk plot

is shown in Fig. 1(a). The broken line is that for the breakdown of the 'normal' ES complex, and the incursion of ESS leads to deviations from this line at high substrate concentrations.

Fig. 1. A curved Lineweaver-Burk plot (continuous line), interpreted in terms of (a) activation by excess of substrate (broken line) or (b) undercorrection for enzyme blank (broken line).

Wolf *et al.* (1964) derive, on the basis of the scheme presented above, 'best-fit' values for k_0 and K_m , from the Michaelis-Menten equation:

$$v_0 = k_0[E_0][S_0]/(K_m + [S_0])$$

for each of the pathways, involving the binary complex (k_0 and K_m) and the ternary one (k'_0 and K'_m). These values are: k_0 0.0062 sec.⁻¹ and K_m 16 mM; k'_0 0.07 sec.⁻¹ and K'_m 654 mM. For the corresponding ethyl ester, Wolf & Niemann (1963*b*) give k_0 0.013 sec.⁻¹ and K_m 96 mM, though Wolf *et al.* (1964) subsequently point out that the enzymic hydrolysis of this ester may be subject to activation by excess of substrate. If this is true, then (cf. the arguments for the methyl ester in Wolf *et al.* 1964) the K_m for the 'normal' binary-complex pathway would be lower than the quoted value. In conflict with these results for the ethyl ester, Inagami & Mitsuda (1964) report k_0 0.066 sec.⁻¹ and K_m 410 mM, and assert that activation by excess of substrate was not observed in their experiments.

The values of K_m for the methyl and ethyl esters of *N*-acetyl glycine given by Niemann and his co-workers for the binary-complex pathway seemed to us unreasonably low (see below), and the reaction of α -chymotrypsin with the ethyl ester was reinvestigated. The enzyme-catalysed hydrolysis of *N*-acetyl glycine thioethyl ester is also reported. A preliminary communication of some of this work has appeared (Ingles & Knowles, 1965).

MATERIALS AND METHODS

***N*-Acetyl glycine ethyl ester.** *N*-Acetyl glycine was esterified by the method of Brenner & Huber (1953). The crude product was purified by three distillations at reduced pressure to give a white crystalline solid, m.p. 46.0–47.5°. Wolf & Niemann (1963*b*) give m.p. 47.0–48.5°. Recrystallization from light petroleum (b.p. 40–60°) did not improve the melting point (Found: C, 49.57; H, 7.60; N, 9.15. Calc. for C₈H₁₁NO₃: C, 49.6; H, 7.58; N, 9.65%).

***N*-Acetyl glycine thioethyl ester (ethyl acetamidoethane-thiolate).** *N*-Acetyl glycine (1.0 g., 1 mole), dried at 100° under high vacuum for 1 hr., was added to ethanethiol (5.5 ml., 8.5 moles) cooled in an acetone–solid CO₂ bath to –20°. Dicyclohexylcarbodi-imide (1.91 g., 1.1 moles) was dissolved in ethanethiol (5.5 ml.), precooled in the acetone–solid CO₂ bath and the two solutions were mixed. The reaction mixture was stirred at –20° for 1 hr. and then slowly brought up to room temperature and stirred for 4 hr. Ethyl acetate (50 ml.) was added and the white precipitate of dicyclohexylurea filtered off. The filtrate was dried over MgSO₄ and the solvent then distilled off under reduced pressure to leave a pale-yellow oil. This oil was exhaustively extracted with boiling light petroleum (b.p. 40–60°), white crystals (m.p. 60–61.5°) forming on cooling the petroleum extracts (yield 59%) (Found: C, 45.1; H, 6.9; N, 8.9; S, 20.2. C₈H₁₁NO₂S requires: C, 44.7; H, 6.8; N, 8.7; S, 19.9%). The ultraviolet spectrum (in ethanol) showed a peak with $\lambda_{\max}$ 232 m μ (log ϵ 3.61).

α -Chymotrypsin. This was a salt-free freeze-dried powder from three-times-crystallized α -chymotrypsin, purchased from Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks. The titration of α -chymotrypsin by the method of Schonbaum, Zerner & Bender (1961) with *N*-cinnamoylimidazole was used to obtain the operational normality of the enzyme solutions used. The enzyme preparation employed was found to be 81% active on a weight basis, assuming a molecular weight of 25 000.

Indole. This was reagent grade from British Drug Houses Ltd., Poole, Dorset.

Kinetics. All kinetic runs were carried out in water at 25 ± 0.1°, 0.1 M with respect to NaCl, by using a pH-stat according to the method of Knowles (1965*a*). Unless otherwise stated the pH at which the runs were done was 7.90. Values of the kinetic parameters were obtained from the raw data by the computational procedures described in Knowles (1965*a*).

RESULTS

The hydrolysis rates of *N*-acetyl glycine ethyl ester and its thiol analogue, in the absence of enzyme, were first-order in both substrate and OH⁻ ion over the whole concentration range employed. The second-order rate constants for the OH⁻ ion-promoted hydrolyses were calculated from the slopes of plots of v_0 versus [OH⁻] and are, for *N*-acetyl glycine ethyl ester and *N*-acetyl glycine thioethyl ester, 0.94 and 0.89 M⁻¹sec.⁻¹ respectively.

Corrections for the spontaneous rate of substrate

Fig. 2. Lineweaver-Burk plot for the α -chymotrypsin-catalysed hydrolysis of *N*-acetyl glycine ethyl ester at pH 7.90 in 0.1 M NaCl at 25°, before correction for the enzyme-blank reaction.

Fig. 3. Lineweaver-Burk plot for the α -chymotrypsin-catalysed hydrolysis of *N*-acetylglycine thiolethyl ester at pH 7.90 in 0.1 M-NaCl at 25°. The continuous line is drawn without inclusion of the two points at lowest $[S_0]$ (see the text).

hydrolysis were applied to each of the initial velocities for the enzyme-catalysed reactions computed from the pH-stat traces.

The Lineweaver-Burk plots for the ethyl and thiolethyl esters obtained by using initial velocities corrected for the substrate-blank reaction (v_0) are shown in Figs. 2 and 3 respectively. The plot for the oxygen ester is appreciably curved, whereas that for the thiol ester departs from linearity only at the lowest $[S_0]$ values. A plot of v_0 versus $[S_0]$ (Fig. 4) shows that the graph is approximately linear at the lower $[S_0]$ values and in both cases has a positive intercept on the v_0 axis. That is, acidic products are being produced in the absence of substrate. This acid liberation must be ascribed to an 'enzyme-blank' reaction.

Kinetic runs were accordingly performed on aqueous solutions containing enzyme alone. This investigation showed: (a) that the enzyme-blank rate is directly proportional to the concentration of enzyme $[E_0]$ at least in the range 10–100 μM , with a pseudo-first-order-rate constant of approx. $1 \times 10^{-3} \text{ sec.}^{-1}$ [in agreement with this, Hofstee (1965) has shown that the autolysis rate of α -chymotrypsin is proportional to $[E_0]$ in the range 4–20 μM]; (b) that the enzyme-blank rate is markedly decreased in the presence of 2 mM-*N*-acetyl-L-tryptophan, a known inhibitor of the enzyme (Foster & Niemann, 1955);

Fig. 4. Plot of v_0 versus $[S_0]$ for the α -chymotrypsin-catalysed hydrolysis of *N*-acetylglycine ethyl ester (●) and *N*-acetylglycine thiolethyl ester (○) at pH 7.90 in 0.1 M-NaCl at 25°.

the rate is decreased to a much smaller extent in the presence of 2 mM-*N*-acetylglycine.

The results obtained in these enzyme-blank studies are in reasonable agreement with the values obtained from the intercepts of the plots of v_0 versus $[S_0]$ for both the ethyl and thiolethyl esters. Thus the pseudo-first-order enzyme-blank rate constants (pH 7.90, 25°, 0.1 M-sodium chloride, $[E_0]$ 30 μM) are: from direct titration of enzyme alone, $1.0 \times 10^{-3} \text{ sec.}^{-1}$; from the intercept for the ethyl ester (Fig. 4), $0.81 \times 10^{-3} \text{ sec.}^{-1}$; from the intercept for the thiolethyl ester (Fig. 4), $1.11 \times 10^{-3} \text{ sec.}^{-1}$.

The initial velocities of the enzyme-catalysed runs with the ethyl and thiolethyl esters were corrected for this enzyme-blank rate by assuming [see (a) above] that the enzyme-blank rate is proportional to the concentration of free enzyme $[E_f]$. Values of $[E_f]$ were calculated for a given substrate concentration from the equation:

$$\frac{[E_f][S]}{[E_{\text{total}}] - [E_f]} = K_m$$

an approximate K_m value having first been obtained from the Lineweaver-Burk plot by using only the

points for high substrate concentration, where the velocities are least perturbed by the enzyme-blank rate. A knowledge of $[E_t]$ together with the value of the intercept on the plot of v_0 versus $[S_0]$, which gives the enzyme-blank rate when $[E_t]$ equals $[E_{\text{total}}]$, enabled the initial velocities to be corrected for the enzyme blank. A corrected Lineweaver-Burk plot gave a more accurate value for K_m that was then re-used in the above equation. The cycle was repeated until K_m did not change by more than 3% round the cycle (in all cases two iterations were sufficient to meet this criterion).

The results obtained for *N*-acetylglucine ethyl ester at substrate concentrations lower than 10mM required enzyme-blank corrections that amount to 40% or more of the total observed velocity. The probable error in the corrected values for these points is therefore rather large, and only points arising from runs where $[S_0]$ is higher than 10mM are included in the final Lineweaver-Burk plot shown in Fig. 5. This procedure is entirely valid, since we have shown that the curvature of the Lineweaver-Burk plots is due to non-correction for the significant enzyme-blank reaction. The most accurate values for the kinetic parameters will therefore be obtained from the points relating to the higher substrate concentrations. For the thiolethyl ester, only the two least concentrated points (0.6 and 0.18mM) require very high enzyme-blank corrections, and these two points are not included in the final Lineweaver-Burk plot for this compound. The kinetic constants for the two substrates

are: for *N*-acetylglucine ethyl ester, K_m 387 ± 47 mM and k_0 $0.039 \pm 0.004 \text{ sec.}^{-1}$; for *N*-acetylglucine thiolethyl ester, K_m 41.0 ± 6.2 mM and k_0 $0.233 \pm 0.033 \text{ sec.}^{-1}$. Thus the probable errors for the oxygen ester are high. These errors are precision estimates derived from the Lineweaver-Burk plot, and do not represent the true accuracy of the values. Any very high K_m value will be subject to large errors (that is, when the intercept on the $1/[S_0]$ axis is close to the origin), but in this case of a very poor substrate, because the points at substrate concentrations above 200mM are subject to substrate-blank corrections of more than 25%, and those below 20mM are subject to enzyme-blank corrections of more than 25%, there is a greater uncertainty in the values. These complications mean that values of 400 ± 100 mM for K_m , and $0.04 \pm 0.01 \text{ sec.}^{-1}$ for k_0 , are probably more reasonable from the standpoint of accuracy.

To show that the α -chymotrypsin-catalysed hydrolysis of *N*-acetylglucine ethyl ester is taking place at the active site of the enzyme, inhibition studies must be performed. Inagami & Mitsuda (1964) have studied the above reaction in the presence of the competitive inhibitor indole. They find the inhibition to be fully competitive with an inhibition constant, K_i , of 1.0mM. We have confirmed that indole is a fully competitive inhibitor of the enzyme-catalysed hydrolysis of the ethyl ester, and find K_i to be 1.9mM. This value, and that of Inagami & Mitsuda (1964), compare reasonably well with that of Foster & Niemann (1955), of 0.8mM, determined against the specific substrate *N*-nicotinyl-L-tryptophanamide.

DISCUSSION

Almond & Niemann (1960), in a paper describing the consequences of systematic error in the kinetic analysis of enzyme reactions, point out that the phenomenon of activation by excess of substrate can be simulated by two situations: undercorrection for the spontaneous rate of hydrolysis of substrate, and undercorrection for the enzyme blank (this latter was first discussed by Martin & Niemann, 1957). The first of these possibilities is not important in the present cases, since the substrate-blank rates have been accurately determined. It is with the second possibility, that of undercorrection for enzyme blank, that we are concerned here. As has been pointed out, it is apparent from the plots of v_0 versus $[S_0]$ for *N*-acetylglucine ethyl and thiolethyl esters that there is a finite and significant rate of liberation of acid when $[S_0]$ is zero. The indications that this is due to an enzyme-blank reaction are: (a) the intercepts for the two esters (the kinetic parameters of which differ markedly) are very close; (b) these values are close to the rate of

Fig. 5. Lineweaver-Burk plot for the α -chymotrypsin-catalysed hydrolysis of *N*-acetylglucine ethyl ester at pH 7.90 in 0.1M-NaCl at 25°, before (●) and after (○) correction for the enzyme-blank reaction.

acid liberation from aqueous solutions of α -chymotrypsin in the absence of substrate; (c) the effect of undercorrection for enzyme blank (in this case, the complete lack of correction) as a systematic error is to simulate activation by excess of substrate (Almond & Niemann, 1960), which is what has been previously proposed to account for the behaviour of the ethyl ester (Wolf *et al.* 1964). Fig. 1(a) demonstrates the effect that substrate activation is expected to have on the 'true' (broken) Lineweaver-Burk line. The alternative interpretation is shown in Fig. 1(b), which represents the deviation from the 'true' (broken) line at low $[S_0]$ values, arising from the failure to correct for the enzyme blank. Thus, if a Lineweaver-Burk plot is curved as in Fig. 1, for substrate activation (or undercorrection for substrate blank) the points most reliably representing the breakdown of the binary ES complex are those at low $[S_0]$ (Fig. 1a), whereas if the curvature is caused by undercorrection for enzyme-blank reaction the most reliable points are those at high $[S_0]$ (Fig. 1b). Clearly a curved Lineweaver-Burk plot has no unique interpretation unless other positive evidence can be adduced to distinguish between these possibilities. In the present case, such evidence is available, and genuinely linear Lineweaver-Burk plots result after correction for enzyme blank.

It is, however, from exactly similar data that Wolf *et al.* (1964) have postulated the incursion of activation by excess of substrate, and their work must now be examined. These authors state: 'activation by excess substrate also can be simulated by overcorrection (*sic*) for the enzyme-blank reaction (Almond & Niemann, 1960). However, for substrates of the type under consideration, Wolf & Niemann (1963a) concluded that the enzyme-blank reaction was insignificant and consequently no such correction was applied'. That is, they rely on the observation of Wolf & Niemann (1963a) that '... no correction for enzyme-blank was required'.

Now, apart from the fact that Almond & Niemann (1960) correctly state that it is undercorrection for enzyme blank that simulates substrate activation, not overcorrection, as subsequently quoted by Wolf *et al.* (1964), the conclusion that no correction for enzyme blank is required is directly contradicted by the latter workers' own data. Plots of v_0 versus $[S_0]$ (similar to Fig. 4) that use the data in Wolf *et al.* (1964) for *N*-acetyl- and *N*-propionyl-glycine methyl esters show intercepts on the v_0 axis at $[S_0]$ equals 0, corresponding to significant enzyme-blank rates. Indeed, quantitatively, these values correspond to pseudo-first-order enzyme-blank rate constants of $1.23 \times 10^{-3} \text{ sec.}^{-1}$ and $1.02 \times 10^{-3} \text{ sec.}^{-1}$ for the two esters. The values are in surprisingly good agreement with the values obtained in the present study.

By using these values for the enzyme-blank rate constants derived from their data, the v_0 values of Wolf *et al.* (1964) were corrected for enzyme blank by the method described in the present work. {For the lowest $[S_0]$ used by Wolf *et al.* (1964) the enzyme blank amounts to 66% of the observed rate.} The Lineweaver-Burk plot (Fig. 6) shows the striking effect of correction for the enzyme blank in the case of *N*-acetylglycine methyl ester. The revised kinetic parameters for *N*-acetyl- and *N*-propionyl-glycine methyl esters are shown in Table 1. The dramatic consequences of effectively assuming that Fig. 1(a) is correct, in a situation represented by Fig. 1(b), are readily apparent from this Table.

There are several other pieces of information that support the contention that substrate activation, as proposed by Wolf *et al.* (1964), does not occur in α -chymotrypsin-catalysed hydrolyses.

(a) The Lineweaver-Burk plot for *N*-acetylglycine thioethyl ester before correction for the enzyme blank (Fig. 3) is linear down to substrate concentrations of 2mM, whereas the plot for the oxygen ester (Fig. 2) is appreciably curved even at $[S_0]$ 20mM. It seems unlikely that two substrates so alike in steric requirements could show such different behaviour if the theory of substrate activation (Wolf *et al.* 1964) is correct. If non-correction for the enzyme blank is the reason for curved Lineweaver-Burk plots, the difference noted

Fig. 6. Lineweaver-Burk plot for the α -chymotrypsin-catalysed hydrolysis of *N*-acetylglycine methyl ester (data of Wolf *et al.* 1964), before (●) and after (○) correction for the enzyme-blank reaction.

Table 1. *Uncorrected and corrected kinetic parameters for the α -chymotrypsin-catalysed hydrolysis of N-acetyl- and N-propionyl-glycine methyl esters*(a) Uncorrected, from Wolf *et al.* (1964), for the binary (ES)-complex pathway; (b) corrected (see the text).

Substrate	K_m (mM)		k_0 (sec. ⁻¹)	
	(a)	(b)	(a)	(b)
N-Acetylglycine methyl ester	16	300	0.0062	0.046
N-Propionylglycine methyl ester	9	170	0.003	0.036

Table 2. *Kinetic parameters for N-acetyl-L-amino acid methyl ester substrates*References: 1, present work; 2, Waite & Niemann (1962); 3, Jones *et al.* (1965); 4, Jones & Niemann (1962); 5, Hein, Jones & Niemann (1962); 6, personal communication from the co-workers of the late Professor Carl Niemann; 7, Knowles (1965a).

Amino acid	k_0/K_m (sec. ⁻¹ M ⁻¹)	K_m (mM)	k_0 (sec. ⁻¹)	Reference
Glycine*	0.0098	387	0.038	1
Valine	1.35	112	0.151	2
Alanine	1.72	739	1.27	3
α -Aminobutyric acid	19.6	53.5	1.05	3
Norvaline	265	10.2	2.7	4
Norleucine	1250	6.7	8.39	3
Leucine	1590	2.9	4.6	5
α -Amino-octanoic acid	2120	2.94	6.23	3
Methionine	2300	8.1	18.6	6
α -Aminoheptanoic acid	8170	1.64	13.4	3
Phenylalanine	42000	1.25	52.5	3
Cyclohexylalanine	80000	0.19	15.2	3
Tyrosine	365000	0.32	117	6
Tryptophan†	420000	0.117	49	7

* Ethyl ester: the methyl ester has not been determined in 0.1 M-salt.

† Ethyl ester: the methyl ester has not been determined at pH 7.9.

between the oxygen and thiol ester is readily explained. Since the thiol ester has a much lower K_m and a higher k_0 , the enzyme-blank correction is less significant for two reasons: (i) when K_m is low the enzyme is protected for more of the time against autolysis, in the form of the ES complex, so that the concentration of free enzyme, and hence the enzyme-blank rate, is lower; (ii) the observed velocities are greater when k_0/K_m is larger (see Fig. 4). This also explains why Wolf *et al.* (1964) failed to observe non-linearity in their Lineweaver-Burk plots for α -N-methanesulphonyl-, α -N-picolinyl-, α -N-nicotinyl-, α -N-isonicotinyl- and α -N-tetrahydrofuroyl-glycine methyl esters. The k_0/K_m ratios for these esters are too high for the enzyme-blank reaction to become appreciable even at the lowest substrate concentrations they employed (5 mM in most cases).

(b) Inagami & Mitsuda (1964) found that N-acetylglycine ethyl ester obeys Michaelis-Menten kinetics over the whole range of substrate concentrations they studied (12–1500 mM). Fig. 2 shows that the Lineweaver-Burk plot for this ester before

correction for the enzyme blank is not significantly deviant from linearity at the lowest $[S_0]$ employed by Inagami & Mitsuda (1964). It is significant that our results for the ethyl ester are in good agreement with those of these workers (K_m 410 mM and k_0 0.066 sec.⁻¹). Our k_0 value is probably more reliable than that of Inagami & Mitsuda (1964) since they obtained their result by extrapolation of data obtained at lower pH values.

(c) In the light of recent suggestions as to the origin of the binding specificity of α -chymotrypsin (Hymes, Robinson & Canady, 1965; Knowles, 1965b), it is very unlikely that the substrate N-acetylglycine methyl ester has a K_m as low as 16 mM. It is apparent (Knowles, 1965b) that, for most of the substrates of α -chymotrypsin listed in Table 2, the relative binding constants are not being determined by the steric limits of the active site, but rather by the relative hydrophobic character of the substrates. Since the glycine substrates are of such high water-solubility (the highest of those quoted in Table 2) it is improbable that these derivatives

should bind much more effectively than the more hydrophobic higher members of the series. Jones, Kunitake, Niemann & Hein (1965) have suggested that the large difference in K_m between *N*-acetylalanine methyl ester (K_m 739mM) and its glycine analogue (K_m 16mM) can be explained in terms of a greater number of possible binding modes for the glycine substrate only. However, since, in the view of Hein & Niemann (1962) non-productive modes of binding are possible and significant for substrates other than just glycine derivatives, this difference is a very large one (nearly 50-fold) to be accounted for in this way.

The postulate of non-productive binding modes can, however, accommodate the relatively minor deviations from the trend of Table 2. Thus, though the order is basically determined by hydrophobic character, minor inversions (e.g. the alanine/glycine K_m ratio 739mM:387mM) are readily accounted for by the alternative binding-mode postulate.

Some comment is necessary on the values of the kinetic parameters for the enzyme-catalysed hydrolysis of *N*-acetyl glycine thiolethyl ester.

First, the K_m value is almost an order of magnitude less than the oxygen analogue. Zerner & Bender (1964) suggest that the chymotrypsin-catalysed hydrolysis of *N*-acetyl glycine ethyl ester follows the mechanistic scheme:

and that the formation of the acyl-enzyme (k_{+2}) is largely rate-determining. This implies that:

$$k_0 = k_{+2} \text{ and } K_m = (k_{-1} + k_{+2})/k_{+1}$$

Since there are strong indications (Zerner & Bender, 1964) that $k_{+2} \ll k_{-1}$, K_m becomes k_{-1}/k_{+1} , i.e., K_s , the dissociation constant of ES. Since also the k_0 value for the thiol ester (0.24sec.⁻¹) is much less than the known k_{+3} for *N*-acetyl glycyl-chymotrypsin (2.29sec.⁻¹; Zerner & Bender, 1964), k_{+2} must still be rate-determining for this ester, and K_m will be K_s . That is, the thermodynamic dissociation constant of ES for the thiol ester is an order of magnitude less than that for the oxygen ester. This bears out the arguments of Hein & Niemann (1962) and Knowles (1965b) that ρ_3 - R_3 interactions (Hein & Niemann, 1961) (that is, interactions between R_3 in the amino acid derivative $R_1 \cdot NH \cdot CHR_2 \cdot COR_3$ and its complementary locus on the enzyme) do contribute significantly to the value of K_s . The view of Knowles (1965b) is also borne out by the fact that the thiol ester is appreciably less water-soluble than its oxygen analogue.

Secondly, the k_0 value for the thiol ester is about six times that of the oxygen compound. This is of

particular interest in view of the fact that the base-catalysed rates of reaction of thiol and oxygen esters are usually within a factor of 2, and the acid-catalysed rates of hydrolysis of thiol esters are usually at least an order of magnitude lower than those for their oxygen analogues (Bender & Connors, 1961). This indicates that there is not an electrophilic component to the catalysis of the acylation step, at least of the type involved in hydronium ion catalysis. This is in agreement with the view of Bender and co-workers (see Bender & Kezdy, 1964, and references therein) that the transition states for the acylation and deacylation steps both resemble the reactions of nucleophiles with the corresponding substrates. The fact that k_{+2} for the thiol ester is actually higher than that for the oxygen ester may be significant. It is known that thiol esters are much more susceptible to nitrogen and carbon nucleophiles than are oxygen esters (Bruice, 1959; Lienhard & Jencks, 1965), and, since current views on the mechanism of chymotrypsin (Bender & Kezdy, 1964) favour an oxygen nucleophile (serine) with a nitrogen general base (histidine), it may be that thiol esters are rather more susceptible than oxygen esters to nitrogen general bases, as well. There is, unfortunately, no evidence on this point.

In summary, therefore, it appears that, even for the poorest *N*-acyl-amino acid substrates of α -chymotrypsin, activation by excess of substrate does not occur. Apart from some examples of non-competitive inhibition (see, e.g., Huang & Niemann, 1953) that may arise from ternary complexes of the ESI type, the action of this enzyme appears to be limited to the binary-complex pathway.

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