The Binding of Inhibitors to α -Chymotrypsin

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1. The binding of three competitive inhibitors, *N*-acetyl-D-tryptophan, *N*-acetyl-L-tryptophan and *N*-acetyl-D-tryptophan amide, to α -chymotrypsin was studied over the pH range 2.20–9.65 by the technique of equilibrium dialysis. 2. Within the limits of the experimental method, the binding of the uncharged amide inhibitor is independent of pH over the range investigated. 3. The binding of each of the enantiomeric acids is dependent on the ionization of a group on the free enzyme, of apparent pK_a 7.3. 4. It is shown that the ionizing group results in the active site of the enzyme developing a net negative charge above pH 7.3. 5. The enzyme groups responsible are tentatively identified, and the significance of the binding constants with respect to the enzymic catalysis is discussed.

Since the first breakdown of a pH-‘activity’ curve into the constituent pH-dependences of individual kinetic parameters by Hammond & Gutfreund (1955), the determination or deduction of the pH-dependences of the rates of single steps in enzymic processes has been exploited, notably by Bender and co-workers for α -chymotrypsin (e.g. see Bender, Clement, Kezdy & Heck, 1964a). The present paper concerns an investigation of the binding equilibrium between α -chymotrypsin and some charged and uncharged specific competitive inhibitors. If, as may reasonably be assumed, the thermodynamic equilibrium between an enzyme and a competitive inhibitor that is a substrate analogue closely parallels the first step (or steps) of the enzymic reaction with substrate, then information from inhibitor-binding studies will be applicable to the relevant part of the enzyme-catalysed reaction. Data exist, of course, for the corresponding process for substrates (K_s), but these suffer from the disadvantages of being experimentally less accessible, liable to perturbations arising from the kinetics and mechanism of the catalytic reaction, and limited to a narrower pH range. Inhibition constants (K_i) determined kinetically suffer from similar limitations, and may vary not only with the substrate against which they are determined but also in complicated ways with the pH (Platt & Niemann, 1963). Therefore in the present work the variation of K_i with pH has been investigated by the method of equilibrium dialysis, so that the K_i value is not perturbed by the kinetic vagaries of a substrate. The inhibitors chosen were *N*-acetyl-D-tryptophan amide, *N*-acetyl-L-tryptophan and *N*-acetyl-D-tryptophan. All of these are known to be competitive inhibitors (Foster & Niemann,

1955b; Foster, Shine & Niemann, 1955) of α -chymotrypsin (the K_i values being determined kinetically with *N*-nicotinyl-L-tryptophan amide or *N*-acetyl-L-tryptophan amide as substrate). It was hoped that the behaviour of the D- and L-acids would manifest any differences in binding specificity (Bender, Kezdy & Gunter, 1964b) between the two enantiomers, and that differences between the behaviour of the D-acid and the D-amide would indicate the nature of the charge at the active site of α -chymotrypsin. Vaslow & Doherty (1953) have reported experiments somewhat similar to those reported below; they used α -chymotrypsin and *N*-acetyl-3,5-dibromo-L- and -D-tyrosine. The pH range (4–7.5) used by these workers was narrower than that (2.20–9.65) studied in the present work and did not include pH values below the pK_a of the carboxyl group of the inhibitors (approx. 3.5). Moreover, *N*-acyl-tryptophan derivatives have only one pK_a (at about 3.6); *N*-acetyl-3,5-dibromotyrosine has a (phenolic) pK_a at about 6.5 in addition to that of the carboxyl group.

MATERIALS

Bovine α -chymotrypsin was purchased from Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks. It was a three-times-crystallized product, salt-free and freeze-dried. Titration of active enzyme by the procedure of Schonbaum, Zerner & Bender (1961) gave 79–81% activity on a weight basis, assuming a molecular weight of 25 000; the ash content was 1% and moisture content 8.2%.

[G-³H]Acetic anhydride was supplied by The Radiochemical Centre, Amersham, Bucks. For the preparation of the D-tryptophan derivatives, 3.7 mg. of this material (25 mc) was diluted with 5.5 ml. of redistilled acetic anhydride. That used for the acetylation of L-tryptophan was

0.7 mg. (25 mc) dissolved in 0.4 ml. of benzene, similarly diluted with unlabelled material.

D- and L-Tryptophan were supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks. The D-acid was recrystallized from aq. 50% (v/v) ethanol, after charcoal decolorization.

N [G- ^{3} H]-Acetyl-D-tryptophan was prepared as follows. D-Tryptophan (2.95 g.) was treated with [G- ^{3} H]acetic anhydride (3.5 ml.) according to the procedure of du Vigneaud & Sealock (1932). After charcoal decolorization and two recrystallizations from water, N [G- ^{3} H]-acetyl-D-tryptophan (2.1 g., 66%) was obtained. It had m.p. 189–190°, $[\alpha]_D^{25}$ –30.3° (c 1 in water containing an equivalent amount of NaOH) and 543 660 counts/sec./m-mole.

N [G- ^{3} H]-Acetyl-L-tryptophan was prepared as above and had m.p. 189°, $[\alpha]_D^{25}$ +28° and 187 450 counts/sec./m-mole; du Vigneaud & Sealock (1932) give m.p. 189–190° and $[\alpha]_D^{25}$ +29°.

N [G- ^{3} H]-Acetyl-D-tryptophan amide was prepared as follows. D-Tryptophan methyl ester hydrochloride was prepared from D-tryptophan by the method described in Greenstein & Winitz (1961). This ester (2.7 g.) was dissolved in the minimum amount of cold water, the pH was adjusted to about 8 and [G- ^{3} H]acetic anhydride (2 ml.) was added in portions, with stirring, at 0°. After each addition of anhydride, the pH was maintained above 7 by the addition of aq. K_2CO_3 solution. On standing at 4° for 1½ hr., N [G- ^{3} H]-acetyl-D-tryptophan methyl ester separated out. Recrystallization from ethyl acetate gave white crystals (2.4 g., 87%) with m.p. 152–153°. Huang & Niemann (1951) give m.p. 152.5°. N [G- ^{3} H]-Acetyl-D-tryptophan methyl ester (2.4 g.) was ammonolysed according to the procedure of Fruton & Bergmann (1942). After recrystallization of the product from ethanol to constant rotation, N [G- ^{3} H]-acetyl-D-tryptophan amide (1.9 g., 83%) was obtained, and had m.p. 192°, $[\alpha]_D^{25}$ –18.5° (c 2 in methanol) and 543 660 counts/sec./m-mole. Huang & Niemann (1951) give m.p. 192–193° and $[\alpha]_D^{25}$ –19°.

All buffers were made up with A.R. reagents and deionized water, and were normally 0.1 M in each component.

The dialysis membranes were cut from Visking dialysis tubing. Before use, the material was repeatedly simmered in fresh distilled water for 5–10 hr. until the hot liquid no longer had a sulphurous smell. The membranes were stored under distilled water, and before use were soaked in the appropriate buffer solution for at least 1 hr.

2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)-benzene were scintillation-grade chemicals supplied by Thorn Electronics Ltd., Polworth, Surrey.

METHODS

Equilibrium dialysis. A small-scale equilibrium-dialysis apparatus was constructed from a cylindrical block of Perspex 7.4 cm. long $\times$ 2.4 cm. diam. Details are shown in Fig. 1. The two halves are bolted (*E*) together and the joint, across which the dialysis membrane (*A*) is fitted, is sealed by a pair of polytetrafluoroethylene gaskets (*B*). Each chamber is filled through its vent (*C*) by using an Agla syringe with micrometer head, and each has a 'bubble cavity' (*D*) that allows the air bubbles to collect at a point from which they are easily expelled. The apparatus is fixed to a bracket carrying a pivot (*G*), which enables the whole cylinder to be rocked from side to side by an electric

Fig. 1. Equilibrium-dialysis apparatus: *A*, membrane; *B*, gasket; *C*, vent; *D*, bubble cavity; *E*, bolt; *F*, stirring marble; *G*, pivot.

motor. The movement of the glass balls (*F*) within each of the shaped chambers thus produces very efficient stirring on each side of the membrane. The volume of liquid held by each chamber can be adjusted by choice of an appropriate ball, and in this apparatus is 1.40 ± 0.02 ml. each side.

The time required for equilibration was determined by placing radioactive inhibitor (approx. 5 mm) into one chamber only and buffer into the other. It was found that the inhibitor became equally distributed across the membrane in less than 10 hr. at approx. 5° (the temperature of all experiments was $5 \pm 3^\circ$, the whole apparatus being set up in a refrigerator).

In a typical experiment, a weighed amount of enzyme solution in the appropriate buffer (0.1 M) was introduced into one chamber. The concentration of enzyme solutions was determined by titration with *N*-cinnamoylimidazole, according to the method of Schonbaum *et al.* (1961), and was normally about 1 mM after dilution. Approximately equal amounts of a solution of the radioactive inhibitor in buffer were then introduced into each chamber. The final concentration of inhibitor was normally about 5 mM. Finally, both chambers were completely filled with buffer. The apparatus was put into the refrigerator, the stirrer motor switched on and the system left to equilibrate for 12–16 hr. Then five 0.1 ml. samples were transferred from each chamber, by using an Agla syringe with a micrometer head, into scintillation bottles and made up to 4 ml. with liquid scintillator. The concentrations of inhibitor were then determined by scintillation counting.

Scintillation counting. Samples were counted on equipment of Isotope Developments Ltd., Beenham, Berks., consisting of Liquid Measuring Head 2022 coupled to Scaler 1700 and Coincidence Control Unit 2032. The routine counting procedure consisted of three measurements of 100 sec. each on each sample. Samples were kept in the dark for 24 hr. between preparation and counting. The liquid scintillator used contained 350 ml. of A.R. toluene, 160 ml. of dry ethanol, 1.75 g. of 2,5-diphenyloxazole and 60 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene. It was found that the addition of up to 0.1 ml. of an aqueous buffer solution to this solution produced a small (approx. 10%) and reproducible decrease in the number of counts recorded. The addition of 0.1 ml. of aq. 1 mM-chymotrypsin solution to a sample caused the enzyme to be precipitated, but the

decrease in counting rate was no greater than that produced by a similar amount of water.

General. The following further checks on the precision of the method were made.

(a) Sampling. It was found that the delivery of 0.1 ml. from an Agla syringe was reproducible to better than $\pm 2\%$.

(b) Counting. The accuracy of the scaler is claimed by the manufacturers to be better than $\pm 2\%$. Since at least 10^4 counts were taken in any 100 sec. determination of counting rate, the standard deviation in counting rate for the mean of three counts on the same sample is about $\pm 0.6\%$. The background count was always about 0.7 count/sec. and was neglected.

(c) pH. The pH values of the solutions on each side of the membrane were measured after equilibration and an average value was taken. Except at pH values above 8.5, the difference between the two chambers was less than 0.03 pH unit. Measurements were made on a Radiometer instrument TTT1c with scale expander PHA 630, standardized against British Drug Houses Ltd. Standard Buffer Solutions.

(d) Volume. Since calculation of the enzyme concentration depends on a knowledge of the volume of solution in the chamber, it was necessary to investigate whether any significant osmotic transport into the enzyme chamber was occurring during equilibration. Volume changes as small as 0.001 ml. (0.07% of the chamber volume) could be measured by placing a calibrated capillary tube in the vent of each chamber. No volume change of more than 0.1% was detected, though it is possible that some transfer could have been masked by slight distension of the membrane.

(e) Donnan effect. The small differences in pH and the small volume changes suggest that the Donnan effect was unimportant and was eliminated by the ionic strength (approx. 0.1) of the solutions used.

(f) Buffer type. The observed values of K_i were probably somewhat affected by the nature of the buffer, but as no trends were apparent this was not investigated.

(g) Enzyme inactivation. For runs at pH values above 8.2, the concentration of enzyme was determined (by the method of Schonbaum *et al.* 1961) after the equilibration, to take account of enzyme denaturation and autolysis. It was assumed that the enzyme concentration measured in this way was equivalent to that involved in the binding equilibrium. This assumption may be invalid, but even at pH 9.6 the enzyme activity had only fallen from 80% (on a weight basis) to 72% (mean of three) during the 16 hr. of equilibration.

(h) Amide hydrolysis. From a knowledge of the second-order rate constant for the base-catalysed hydrolysis of *N*-acetyl-L-tryptophan amide (Hawkins, 1965), it was expected that the amount of hydrolysis of the D-amide inhibitor used in this work would be less than 1% in 16 hr. at pH 10. This was checked experimentally by incubating solutions of 5 mM-*N*-acetyl-D-tryptophan amide in carbonate buffer, pH 9.8, at 5° for 20 hr. with and without 1 mM-chymotrypsin. These solutions were compared spectrophotometrically at 306 m μ (Bender *et al.* 1964a) with *N*-acetyl-D-tryptophan and with a fresh solution of *N*-acetyl-D-tryptophan amide. No hydrolysis of the amide could be detected in the presence or absence of enzyme.

(i) Tritium exchange. To check that no loss of ^3H from the inhibitors occurred at the higher pH values, *N*-acetyl-D-tryptophan amide was reisolated after 20 hr. in carbonate

buffer, pH 9.8, at 5°. The specific activity of this material was found to be identical with that of the unincubated material.

(j) Adsorption losses. No loss of radioactive material by adsorption on to membrane, chamber walls or stirring marbles was detected.

RESULTS

From the average of the 15 radioactivity measurements (five samples, counted three times each) of solution taken from each side of the membrane, the K_i (i.e. the dissociation constant of the EI complex) was calculated according to:

$$K_i = ([E_t] - [\Delta I]) [I_0] / [\Delta I]$$

where $[E_t]$ is the total active enzyme concentration (M) as determined by titration with *N*-cinnamoylimidazole, $[I_0]$ is the concentration of inhibitor (M) on the non-enzyme side of the membrane, and $[\Delta I]$ is the difference between the concentrations of inhibitor on each side of the membrane. It is assumed that only one molecule of inhibitor is bound to each protein molecule. The values obtained for *N*-acetyl-L-tryptophan, *N*-acetyl-D-tryptophan and *N*-acetyl-D-tryptophan amide are presented in Tables 1, 2 and 3 respectively. These data are plotted against pH in Figs. 2 and 3. It is apparent that, though K_i for the amide is approximately independent of pH in the pH range 2.20–9.65, K_i for the two acids depends on the ionization of a group of pK_a near neutrality. In the manner of Dixon (1953) it can be shown that a plot of pK_i against pH will result in a graph made up of straight-line sections of integral gradient, which intersect at a pH corresponding to the pK_a of an ionizing group in one of the components or in the EI complex. If at

Table 1. *Values of K_i for the binding of N-acetyl-L-tryptophan to α -chymotrypsin*

Equilibrium dialysis was carried out in 0.1 M-buffer at approx. 5°. $[E_t]$ was approx. 1 mM and $[I_0]$ was approx. 5 mM.

pH	Buffer type	K_i (mM)	pK
2.15	Citrate-phosphate	1.6	2.80
2.77	Citrate-phosphate	3.0	2.52
3.45	Citrate-phosphate	1.4	2.85
4.27	Citrate	2.6	2.58
5.57	Phosphate	2.0	2.70
5.77	Phosphate	0.9	3.05
6.60	Phosphate	2.7	2.57
7.26	Tris-HCl	4.1	2.39
7.61	Tris-HCl	4.1	2.39
7.68	Tris-HCl	5.2	2.28
8.03	Tris-HCl	14.3	1.84
8.27	Tris-HCl	23.4	1.63
8.70	Carbonate	55.4	1.26

an intersection the slope increases with pH increase the pK_a relates to the complex, and if it decreases the pK_a relates to free enzyme or free inhibitor. [Though this may not be a valid argument for K_i values determined kinetically (see Webb, 1963), it is correct for binding constants determined directly, as in the present experiments.] Fig. 4 shows such logarithmic plots for the enantiomeric acid inhibitors, and there is in each case a decrease in slope with rise in pH, implying that the K_i is affected by an ionizing group on the free enzyme or on the free

Table 2. Values of K_i for the binding of *N*-acetyl-D-tryptophan to α -chymotrypsin

Equilibrium dialysis was carried out in 0.1M-buffer at approx. 5°. $[E_i]$ was approx. 1 mM and $[I_0]$ was approx. 5 mM.

pH	Buffer type	K_i (mM)	pK_i
2.20	Citrate-HCl	3.4	2.47
2.75	Citrate-phosphate	2.6	2.58
3.64	Citrate	1.6	2.80
4.55	Citrate	2.0	2.70
5.46	Citrate	1.6	2.80
6.72	Phosphate	1.6	2.80
7.25	Phosphate	3.3	2.48
7.65	Tris-HCl	4.0	2.40
7.75	Tris-HCl	3.0	2.52
7.93	Tris-HCl	6.5	2.19
8.10	Tris-HCl	6.75	2.17
8.20	Tris-HCl	19.5	1.71
8.44	Tris-HCl	13.0	1.89
8.72	Tris-HCl	20.4	1.70

Table 3. Values of K_i for the binding of *N*-acetyl-D-tryptophan amide to α -chymotrypsin

Equilibrium dialysis was carried out in 0.1M-buffer at approx. 5°. $[E_i]$ was approx. 1 mM and $[I_0]$ was approx. 5 mM.

pH	Buffer type	K_i (mM)	pK_i
2.28	Citrate-phosphate	0.4	3.40
2.74	Citrate-phosphate	3.8	2.43
3.43	Citrate-phosphate	4.0	2.40
3.52	Citrate	4.0	2.40
4.22	Citrate	3.9	2.41
4.41	Citrate	2.1	2.68
5.40	Citrate	1.3	2.89
6.35	Phosphate	0.5	3.30
7.09	Phosphate	2.1	2.68
7.70	Tris-HCl	0.6	3.22
8.09	Tris-HCl	0.6	3.22
8.55	Borate	2.8	2.56
8.85	Tris-HCl	0.2	3.70
9.05	Carbonate	0.6	3.19
9.25	Carbonate	3.0	2.52
9.65	Carbonate	3.6	2.44

inhibitor. The change in slope is from 0 to -1 , and the broken lines in Fig. 4 have the theoretical slopes. Since the free inhibitors have only one pK_a (that of the carboxyl group at 3.63; Kezdy, Clement &

Fig. 2. Plot of K_i against pH for the binding of *N*-acetyl-D-tryptophan (●) and *N*-acetyl-D-tryptophan amide (○) to α -chymotrypsin.

Fig. 3. Plot of K_i against pH for the binding of *N*-acetyl-L-tryptophan (●) and *N*-acetyl-D-tryptophan amide (○) to α -chymotrypsin.

Fig. 4. Plot of pK_i against pH for the binding of *N*-acetyl-D-tryptophan (○) and *N*-acetyl-L-tryptophan (●) to α -chymotrypsin. The broken lines have the theoretical slopes of 0 and -1 .

Table 4. Comparison of values of K_i determined directly and kinetically

Values of K_i determined directly were obtained by interpolation in the present study. Values of K_i determined kinetically are taken from Foster & Niemann (1955a).

Inhibitor	pH	K_i (mm)	
		Direct determination	Kinetic determination
<i>N</i> -Acetyl-D-tryptophan	6.9	2.0	1.3
	7.9	6.5	7.5
<i>N</i> -Acetyl-L-tryptophan	6.9	3.0	2.0
	7.9	10.5	10.0
<i>N</i> -Acetyl-D-tryptophan amide	6.9	~ 2.0	1.7
	7.9	~ 2.0	2.3

Bender, 1964) the pK_a near neutrality must relate to an ionizing group on the free enzyme. The results do not permit a very accurate determination of this pK_a , but it is clear (Fig. 4) that the behaviour of the D- and L-acids are closely similar, and that a group on the enzyme with pK_a about 7.3 is affecting the binding of the two acids equally.

The actual values of K_i for the three inhibitors used can be compared with values for the same compounds determined kinetically by Foster & Niemann (1955a) at pH 6.9 and 7.9. Their values are presented in Table 4 and the agreement is seen to be very good. The value of K_m for the ^{18}O -exchange reaction of *N*-acetyl-L-tryptophan (catalysed by α -chymotrypsin at pH 7.9) of 4 mm obtained by Bender & Kemp (1957) is also of the same order as the value of K_i at pH 7.9 from the binding experiments, namely 10.5 mm.

DISCUSSION

The results of the binding experiments reported here indicate the following: (a) the binding of an uncharged competitive inhibitor, *N*-acetyl-D-tryptophan amide, to α -chymotrypsin is independent of pH over the range 2.28–9.65; the variations that are seen (Fig. 2) are probably the result of experimental imprecision and specific buffer effects; (b) the binding of each of two enantiomeric charged inhibitors, *N*-acetyl-D- and -L-tryptophan, depends markedly on pH, and this dependence may be interpreted in terms of an ionizing group on the enzyme having an apparent pK_a approx. 7.3; that this group is on the free enzyme is certain, for acetyltryptophan has no pK_a in this region, and only the ionization of a group on the free enzyme or on the free inhibitor produces a negative change in gradient in the graph of pK_i against pH; (c) the binding of both the D- and L-acids leads to the same value for the apparent pK_a of the ionizing group.

Since the binding of the amide is not affected by variation in pH, the forces contributing to its binding cannot be electrostatic, but are (in agreement

with other evidence) largely hydrophobic and possibly hydrogen-bonding (Knowles, 1965; Bender & Kezdy, 1965). This assumes that any pH-dependent changes in the conformation of the protein, or in its degree of flexibility, do not seriously affect the binding of a specific inhibitor. Hence, whether the inhibitor is bound to a preformed and rigid active site, or whether it effectively creates (or completes) its own site (by an induced-fit mechanism; Koshland, 1959), the constant for the enzyme-inhibitor equilibrium is not affected by pH. This agrees with the results of Bender *et al.* (1964a), who found that the K_m values (for these cases $K_m = K_i$) for *p*-nitrophenyl acetate, *N*-acetyl-L-tryptophan amide and *N*-acetyl-L-phenylalanine amide are roughly independent of pH over the range approx. 6–10. However, the present results are not in accord with the proposal of Stewart, Lee & Dobson (1963), who found that K_m for the non-specific substrate 2,4-dinitrophenyl acetate was dependent on a group on the enzyme of pK_a about 4.

Before considering the enantiomeric acid inhibitors, we must consider the effect of the role of the L-acid as a virtual substrate rather than as a simple inhibitor. Since α -chymotrypsin-catalysed processes include the formation of an acyl-enzyme (Bender & Kezdy, 1964), then, although the equilibrium binding of the D-compounds involves only $\text{E} + \text{I} \rightleftharpoons \text{EI}$, the binding of the virtual substrate (the L-acid) involves $\text{E} + \text{I} \rightleftharpoons \text{EI} \rightleftharpoons \text{AE}$ (where AE is the acyl-enzyme). However, the close similarity in behaviour of the L- and the D-acids, when only the L-acid is a virtual substrate (^{18}O -exchange does not occur with *N*-acylated D-acids; Bender & Kemp, 1957), suggests that any effect arising from the possibility of acyl-enzyme formation with the L-acid is insignificant. In any case, the K_i measured by the equilibrium dialysis method will simply measure (for a virtual substrate) the ratio $[\text{EI} + \text{AE}]/[\text{E}]$ instead of $[\text{EI}]/[\text{E}]$. Since AE formation will be significant only in the region of enzymic activity, the effect would be to decrease K_i at about the pH optimum of the enzyme (approx.

Two problems remain, namely the charge type and identity of the ionizing group on the enzyme. The binding of the D-amide is unaffected by pH, so the possibilities are: (a) that as the pH rises above neutrality the acid inhibitor anions (pK_a 3.63) are repelled by the binding site because of the development of an actual negative charge (or a net negative charge) on the enzyme with pK_a approx. 7.3; (b) that the development of a positive charge on the enzyme as the pH falls below neutrality results in stronger binding of the inhibitor anions owing to an electrostatic interaction between unlike charges. This second possibility is unattractive, since it predicts that the binding of acids at high pH (unaffected by the charge-charge attraction) would be very much poorer than that of the amide. This is unlikely because: (i) it is probable that the major contribution to the binding of substrates and inhibitors to α -chymotrypsin arises from hydrophobic interactions that are not dependent on pH; (ii) it is believed that the contribution to binding of the carboxyl function of such molecules is small (Knowles, 1965) or absent (Zerner & Bender, 1964).

From a calorimetric study of the hydrocinnamate- α -chymotrypsin system, Canady & Laidler (1958) postulated that a group in the active site was 'largely neutralized at pH 7.1 but largely negative at pH 7.9'; similarly, from studies of kinetically determined inhibition constants of charged and uncharged inhibitors, it was proposed that a negative charge developed in the environment of the active site at about pH 7.9 (for earlier references to this proposal see Foster & Niemann, 1955*a*). Both of these suggestions are in accord with postulate (a) above. However, there are no very convincing candidates for an amino acid residue of pK_a 7.3 that is anionic at high pH values. The possible groups are: carboxyl, pK_a 2.2-5.1; thiol, pK_a 8.3-10.1; phenolic hydroxyl, pK_a 9.6-10.5 (Webb, 1963). α -Chymotrypsin has no thiol groups, and a carboxyl or phenolic hydroxyl group would need very substantial perturbation (for which titration studies provide no evidence; Marini & Wunsch, 1963) to have pK_a 7.3. It therefore seems reasonable to suggest that the ionizing group on the enzyme with pK_a 7.3 is a positively charged species below neutrality, which, on loss of a proton, leaves the relevant region of the active site with a net negative charge. This demands the fairly close juxtaposition of (say) two ionizing groups at the active site of the enzyme, one carrying a negative charge over the range of interest (namely above pH 6) and the other, which has pK_a about 7.3, carrying a positive charge in its acid form. Possibilities for the two groups are a carboxyl group (? Asp-194) and an imidazole group (? His-57; Hartley, 1964). The former is adjacent to the serine residue (Ser-195) that is acylated during the catalytic process (Bender & Kezdy, 1964), and the

Scheme 1.

latter is close to the carboxyl function of specific substrates and inhibitors (Schoellmann & Shaw, 1962; Ong, Shaw & Schoellmann, 1965) and tentatively identifiable as the group responsible for the decrease in activity as the pH is lowered below the optimum (Bender & Kezdy, 1964). The situation is shown in Scheme 1, where above pH 7.3 the locus of the active site containing the carboxyl function of the inhibitor is negatively charged and repels the inhibitor anion (this repulsion overcoming the other attractive binding forces); below pH 7.3 this locus has no net charge and the inhibitor binds, and below pH 3.6 the carboxyl groups of both inhibitor and enzyme are protonated and binding is still unperturbed (provided, of course, that $pK_{\text{CO}_2\text{H}}^{\text{inhibitor}} \geq pK_{\text{CO}_2\text{H}}^{\text{enzyme}}$). The uncharged D-amide will be relatively unaffected by the ionization of enzyme, and remain bound throughout, as observed.

Finally, at the pH optimum of this enzyme (approx. pH 8), the substrate has a binding constant five or ten times more favourable than that of the product. According to the above interpretation, the very ionization that leads to the appearance of enzymic activity (that of histidine, pK_a approx. 7) also results in a situation where the product of this catalysis is expelled from the active site.

Note added in proof. At pH values above 9.65, K_i for N-acetyl-D-tryptophan amide rises sharply, indicating an ionizing group on the enzyme of pK_a approx. 9.3.

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