The Binding of Inhibitors to α -Chymotrypsin at Alkaline pH

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1. The binding of the competitive inhibitor *N*-acetyl-D-tryptophan amide to α -chymotrypsin has now been studied at pH values up to 10.6, by the technique of equilibrium dialysis. 2. This binding depends on the ionization of a group on the free enzyme with apparent pK_a 9.3 at 5°. 3. This group is tentatively identified as that responsible for an enzyme conformation change at high pH values, on which the catalytic activity of the enzyme also depends.

In a previous paper (Johnson & Knowles, 1966) we reported the pH-dependence of the inhibition constant K_i of three competitive inhibitors of α -chymotrypsin: *N*-acetyl-D-tryptophan, *N*-acetyl-L-tryptophan and *N*-acetyl-D-tryptophan amide. It was found that K_i for each of the two enantiomeric acids is dependent on the ionization of a group on the free enzyme, of apparent pK_a approx. 7.3, and that K_i for the D-amide was essentially independent of pH over the range investigated (2.28–9.65). Recently, however, it has been found (Himoe & Hess, 1966; Bender, Gibian & Whelan, 1966) that K_m for the substrate *N*-acetyl-L-tryptophan amide with α -chymotrypsin is pH-dependent, and rises sharply above pH 9. Since for this substrate it has been shown that the acylation step is rate-determining (Zerner & Bender, 1964), and K_m therefore equals K_i (the thermodynamic dissociation constant for the enzyme-substrate complex), it became important to extend our earlier measurements (Johnson & Knowles, 1966) above pH 9.65, to ascertain whether the pH-dependent behaviour of K_i of the D-amide differs from or is similar to the behaviour of K_i of the L-amide. The present paper reports these additional data, which show that the variations in thermodynamic dissociation constants for enzyme-inhibitor and enzyme-substrate complexes with pH, run parallel to one another.

MATERIALS AND METHODS

All materials and methods, including checks on enzyme denaturation, tritium exchange and inhibitor solvolysis, were as described by Johnson & Knowles (1966).

RESULTS AND DISCUSSION

The values of K_i for *N*-acetyl-D-tryptophan amide at pH values above 9.65 are presented in

Table 1. These data, together with those for this inhibitor at the lower pH values used previously, and also with the values for *N*-acetyl-D-tryptophan, are plotted against pH in Fig. 1. A logarithmic plot

Table 1. 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. 1mM and $[I_0]$ was approx. 5mM. For data at lower pH values, see Johnson & Knowles (1966).

pH	Buffer	K_i (mM)
9.80	Carbonate	6.7
10.10	Borate-NaOH	11.2
10.10	Borate-NaOH	10.2
10.10	Borate-NaOH	10.4
10.10	Borate-NaOH	7.2
10.30	Borate-NaOH	14.3
10.60	Borate-NaOH	117
11.75	NaOH	∞

Fig. 1. Plot of K_i against pH for the binding of *N*-acetyl-D-tryptophan amide (●) and *N*-acetyl-D-tryptophan (○) to α -chymotrypsin.

of this Figure (according to Dixon, 1953) shows that binding of the acid is affected by an ionizing group of pK_a approx. 7.3 and binding of the neutral amide is affected by the ionization of a group of pK_a approx. 9.3. The direction of the gradient change in the logarithmic plot implies that both these pK_a values relate to the free enzyme or the free inhibitor (Dixon, 1953), and since neither inhibitor has pK_a in the region under consideration (pK_a of *N*-acetyltryptophan is 3.63; Kezdy, Clement & Bender, 1964), both of the curves of Fig. 1 must arise from ionizing groups on the free enzyme.

The interpretation of the origin of pK_a 7.3, observed in the binding of the enantiomeric acids (Johnson & Knowles, 1966), is unaffected by the present experiments. The data are consistent with the creation of a net negative charge in the active site of α -chymotrypsin as the pH rises past neutrality, and the groups responsible for the creation of this net negative charge were postulated to be Asp-194 and His-57 (Hartley, 1964).

The new data presented here show that a neutral, specific, competitive inhibitor of α -chymotrypsin binds progressively less well to the protein above about pH 9.0. (Inspection of a semilogarithmic pH versus K_i plot alone may be misleading: large effects on K_i are only apparent after the pH is quite far beyond the apparent pK_a .) This is in agreement with the finding of Deranleau & Neurath (1966) that the binding to α -chymotrypsin of the inhibitors *N*-1-dimethylaminonaphthalene-5-sulphonyl-D-tryptophan ethyl ester and the corresponding glycine derivative is much less good at pH 10 than at pH 8. The forces which are important for the binding of neutral inhibitors to α -chymotrypsin are hydrophobic interactions (Knowles, 1965) and (largely for orientational purposes) hydrogen bonds (Ingles, Knowles & Tomlinson, 1966). Electrostatic interactions between enzyme and inhibitor cannot, of course, be relevant for the neutral inhibitor molecule. Although it is possible that an ionizing group on the enzyme could lead to a decrease in the hydrophobic character of the active site (for instance, a tyrosine residue could ionize with pK_a about 9.5 and lead to a reduction in the hydrophobic nature of the site), or that deprotonation of an enzyme group could lead to a loss of hydrogen-bonding capacity, neither of these effects is likely to lead to a reduction in the free energy of binding of *N*-acetyl-D-tryptophan amide such that K_i rises from approx. 3 mM to 'infinity' (which, for the method used, means > 100 mM). A much more attractive possibility is that there is a pH-dependent enzyme conformation change, which effectively destroys the substrate- and inhibitor-binding site above about pH 9. For the substrate *N*-acetyl-L-tryptophan amide, for which it is believed that $K_m = K_i$ (Zerner & Bender, 1964), it has been shown

that K_m rises rapidly at high pH values with apparent pK_a approx. 9 (Himoe & Hess, 1966; Bender *et al.* 1966). This means that (at least for *N*-acetyltryptophan derivatives) the pH-dependence of the binding of neutral L-substrates parallels that of neutral D-inhibitors.

The work of G. P. Hess and his group (e.g. Oppenheimer, Labouesse & Hess, 1966) has indicated that the conversion of chymotrypsinogen into the active enzyme is accompanied by the appearance of a new ionizing group, the prototropic equilibrium of which controls the conformation and the activity of the enzyme. From studies of the pH-dependence of the catalytic activity and optical activity of acetylated δ -chymotrypsin, Hess concludes that the ionizing group is the α -amino group of *N*-terminal isoleucine, and that chymotrypsin molecules in which this group is protonated are in a catalytically active conformation, deprotonation of the group resulting in a conformation change to an inactive (chymotrypsinogen-like) isomer. In acetylated δ -chymotrypsin the ionizing group has apparent pK_a 8.3, on the basis of titration studies (at 4°) and optical rotation work (at 12°). For the α -chymotrypsin-catalysed hydrolysis of substrates, the right-hand limb of the bell-shaped profile of k_o/K_m versus pH indicates that the activity of α -chymotrypsin depends on an ionizing group with pK_a between about 8.5 and 9.0 (Bender, Clement, Kezdy & Heck, 1964). Oppenheimer *et al.* (1966) propose that the group with pK_a 8.3 in acetylated δ -chymotrypsin is the same as that which affects the activity of α -chymotrypsin in the high pH region. Moreover, they account for the difference in pK_a by postulating that the substrate induces a conformation change in the enzyme which has the effect of raising the apparent pK_a (into the 8.5–9.0 region). In our experiments, the rules of Dixon (1953) require that the group observed with pK_a 9.3 is on the free enzyme, and that we are not observing any perturbation of this pK_a by inhibitor binding (otherwise a wave would result in the pK_a versus pH plot; Dixon, 1953). If we assume that the group is an amino group, on the basis of the probable heat of ionization (Cohn & Edsall, 1943), the pK_a 9.3 at 5° observed in the present experiments would be about 8.7 at 25° (the temperature of most of the kinetic experiments). This indicates that the group, the ionization of which affects the binding of *N*-acetyl-D-tryptophan amide, is the same as that which affects the binding of substrates (e.g. K_m of *N*-acetyl-L-tryptophan amide; Himoe & Hess, 1966). This is probably the α -amino group of *N*-terminal isoleucine (Oppenheimer *et al.* 1966), which is known to affect the conformation and control the catalytic activity of the enzyme.

The pH-dependence of the binding of charged and

uncharged competitive inhibitors to α -chymotrypsin (this paper, and Johnson & Knowles, 1966) therefore appears to have shown up both the ionizing groups on the enzyme which are believed to be responsible for the pH-dependence of the catalytic reaction.

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