Conformational Equilibria and the Salt Bridge in Chymotrypsin

ALAN R. FERSHT
MRC Laboratory of Molecular Biology, Cambridge, England

δ-Chymotrypsin is stabilized in the active conformation by a salt bridge between the α-ammonium group of Ile 16 and the carboxylate ion of Asp 194. At neutral pH the enzyme exists mainly in the active conformation but at high pH where the salt bridge is deprotonated only 12% of the enzyme takes up the active conformation.

The stabilization energy of the salt bridge is 2.9 kcal/mole. The formation is entropy driven and involves an adverse enthalpy change. The pK_a of Ile 16 is increased from 7.9 when unconstrained in the inactive conformation to 10 when in the salt bridge in the active conformation (*see* Fig. *1*).

I will now briefly describe how these results were obtained. Chymotrypsinogen is activated to chymotrypsin by the tryptic cleavage of the Arg 15-Ile 16 bond (Dreyer and Neurath, 1955; Rovery et al., 1955). X-ray diffraction studies on the enzyme and zymogen indicate that the α-ammonium ion of the Ile 16, which is released on activation, forms a salt bridge with the buried carboxylate ion of Asp 194 and in doing so forms a binding pocket for the hydrophobic regions of specific substrates and inhibitors (Sigler et al., 1968; Freer et al., 1970). There is indirect evidence that similar conformational changes take place in solution. At low pH the salt bridge is intact and the enzyme exists mainly in the active conformation. At high pH the salt bridge is deprotonated and the enzyme reverts to a zymogen-like conformation unable to bind the specific substrates or inhibitors. The transition between the two conformations is governed by an apparent pK_a of about 9 composed of the real ionization constants of Ile 16 and the equilibrium constants for the conformational equilibria (Hess et al., 1970). These ionization and equilibrium constants can be used to determine the energy of the salt bridge. This is of some general interest as electrostatic linkages are of importance in determining tertiary and quaternary structure and there is a lack of experimental values.

We have directly observed the existence and interconversion of the two conformations in α-chymotrypsin by stopped-flow spectrophotometry (Fersht and Requena, 1971) (Fig. *2*). On mixing enzyme incubated at 25° in dilute buffer with an excess of the reversible inhibitor proflavin incubated in a concentrated pH 6.84 buffer, there is a burst of enzyme-proflavin complex formation due to association of the dye with the active conformation; this is followed by a slow first order increase as the inactive conformation becomes active and further combines with the dye. The ratio of the two changes gives the proportion of enzyme in each conformation. Enzyme incubated at high pH gives a negligible burst, corresponding to a low proportion of active conformation, whereas at neutral pH 85% is in the active and 15% in the inactive conformation. The plot (Fig. *3*) of the proportion of active conformation against pH defines an ionization curve of pK_a 8.76. This value in conjunction with the equilibrium constant of 15%–85% gives the pK_a of Ile 16 as 7.94 ± 0.1 in the inactive conformation, i.e., free in solution. However, it is not possible to determine the pK_a of Ile 16 in the salt bridge as the proportion of active enzyme at high pH is immeasurably small by this technique.

δ-Chymotrypsin, unlike α-, binds substrates at high pH and the proflavin binding experiments were repeated for this form of the enzyme (Fig. *4*). At neutral pH about 90% takes up the active conformation; at high pH 12% remains in the active conformation despite the salt bridge being

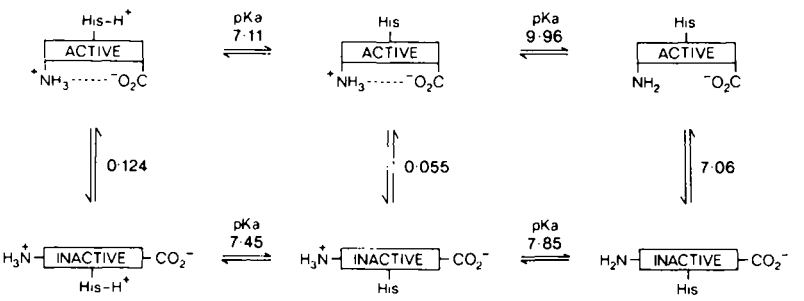

Figure 1. Summary of results for δ-chymotrypsin at 25° and ionic strength 0.1. The active conformation binds specific substrates, the inactive does not. The amino and carboxylate groups shown are those of Ile 16 and Asp 194, respectively. The equilibria on the right are due to the ionization of Ile 16. Those on the left are perturbations due to a histidine ionization.

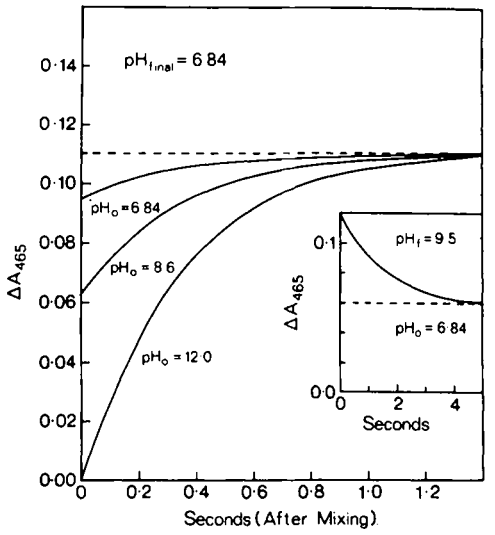

Figure 2. Formation of α-chymotrypsin-proflavin complex followed at 465 nm by stopped-flow spectrophotometry. Enzyme incubated in a dilute buffer (25°, ionic strength 0.1) at pH_0 is mixed with excess proflavin in a concentrated pH 6.84 phosphate buffer to give a final pH of 6.84. *Inset:* The enzyme initially at pH 6.84 jumped with proflavin solution to pH 9.5.

deprotonated. The results are analyzed in terms of two sets of ionizations; an apparent alkaline transition of pK_a 9.08 and a small perturbation of pK_a 7.14. The solid curve is calculated from these and the equilibrium constants. The perturbation at pH 7 is presumably due to a His ionization. For example, His 40 is much closer to Asp 194 in the zymogen than in the enzyme and is expected to

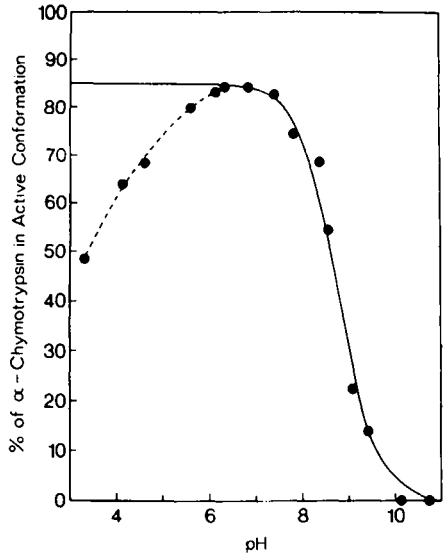

Figure 3. The percentage of α-chymotrypsin in the active conformation as a function of pH at 25° and ionic strength 0.1. The solid line is calculated assuming a pK_a of 8.76 and a maximum of 85% active. The decrease at low pH is due to the progressive ionizations of the carboxyl groups.

have a higher pK_a in the inactive conformation. The pK_a of Ile 16 is a normal 7.85 in the inactive conformation but is increased to 9.96 in the active conformation in the salt bridge.

The combination of exact structural knowledge from X-ray diffraction in conjunction with physical measurements in solution provides a unique opportunity for measuring the stabilization energy of a salt bridge. This is found from the ratio of equilibrium constants at high and neutral pH to be 2.9

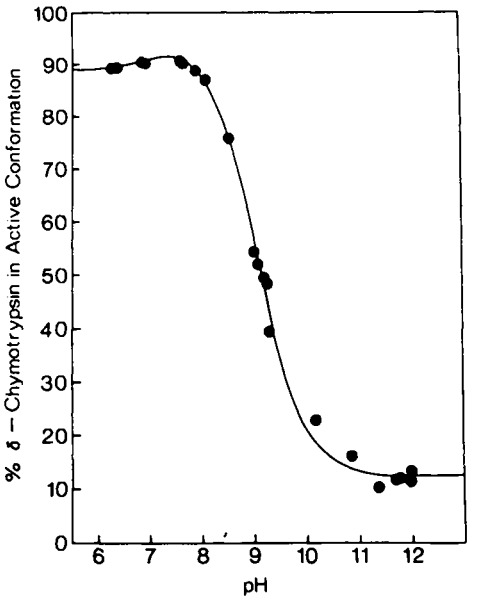

Figure 4. Percent of δ-chymotrypsin in the active conformation as a function of pH at 25° and ionic strength 0.1. The solid line is that calculated for the equilibrium constants in Fig. *1*. The alkaline limb fits an apparent pK_a of 9.08.

kcal/mole. This is a buried salt bridge and is presumably much stronger than a surface one as found in hemoglobin between the α-ammonium ion of one α-chain and the α-carboxylate in the other.

A temperature study shows that the proportion of active conformation increases with increasing temperature. The gross changes on formation of the salt bridge involve a favorable entropy term of 27 eu but an unfavorable enthalpy change of 7 kcal.

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