Refolding Transition of α-Chymotrypsin: pH and Salt Dependence†

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ABSTRACT: It is well known that α-chymotrypsin can exist in two major conformational states, only one of which is active. We have examined the pH (pH 2.0–11.0) and salt (ionic strength 0.01–1.0) dependence of the transition between the active and inactive forms in detail. At low pH (pH 2.0–6.0) the equilibrium is very dependent on salt concentration, with high salt concentrations effectively stabilizing the active conformation. This apparent stabilization is an artifact due to the salt-dependent dimerization of α-chymotrypsin, and our data show that only active species form dimers and higher aggregates. At neutral pH (6.0–8.0) dimerization is absent, yet an ionic strength dependence remains. The effects show no lyotropic order and appear to be due to preferential salt binding to the active conformation at one or possibly a few sites. Above pH 6 (pH 6.0–11.0), the pH dependence can be described by a two-ionization mechanism at all ionic strengths. We report values for all seven equilibrium constants in the proposed mechanism at four ionic strengths (μ = 0.01, 0.05, 0.2, and 1.0). The transition is the first "refolding" transition to be studied at high precision, but, even so, certain decisions about the mechanism must await higher experimental precision not available with present methods.

Despite intense work, many, and perhaps most, of the uniquely enzymatic factors contributing to chymotryptic catalysis are not understood. As usual in kinetics processes, quantitative studies provide qualitative descriptions never clearly established by nonquantitative molecular manipulations. Increasingly, quantitative approaches to chymotrypsin are employed in several laboratories, but these are restricted by the pH-, salt-, and temperature-dependent transitions manifested by the protein. In this paper, we successfully remove most of these complications. In addition, the basic underlying transition has intrinsic interest as a "refolding transition," defined as one in which the activated complex has a greater degree of unfolding than either stable state.

It is well known that there are two major conformational forms of α-chymotrypsin (Hess et al., 1970; Birktoft et al., 1970; Freer et al., 1970; Hess, 1971; Kraut, 1971; McConn et al., 1969; Fersht and Requena, 1971; Fersht, 1972; Ghelis et al., 1970; Garel and Labouesse, 1970; Taylor et al., 1973). Summarizing briefly, the commonly held interpretation of the experimental results is as follows:[1] Only an active form, which predominates at neutral pH values, is able to bind substrates and inhibitors. At pH extremes, an inactive form, which is unable to bind these small molecules and may resemble chymotrypsinogen in structure, predominates. The α-amino group of Ile-16 is inaccessible to solvent and has a pK_a near 10 in the active form because it is involved in an internal ion pair with the Asp-194 carboxylate group. Deprotonation of Ile-16 at high pH or protonation of Asp-194 at low pH destroys this ion association, and the molecule isomerizes to the inactive conformation. In the latter form, the Ile-16 α-amino group is more accessible to solvent and has a pK_a normal for small-molecule models. The interconversion between the active and inactive forms is slow and has a large activation energy, suggesting that a major reorganization of structure accompanies ion-pair breakage. The conformational transition itself may have little direct connection with chymotryptic catalysis, but it provides a useful experimental focus for observing the factors involved in formation and maintenance of the catalytically active structure and an approach to the role of the "buried" ion pair, which is an invariant feature to this large family of enzymes. The studies reported here provide a necessary basis for further work on this problem.

In this paper, we examine the pH and electrolyte dependence of the equilibrium between active and inactive enzyme. Above pH 6, the pH dependence can be described by the two-ionization mechanism previously proposed for δ-chymotrypsin (Fersht, 1972). No significant ionic strength effects are observed at high pH, but near neutral pHs there is a salt effect, probably due to preferential ion binding by the active species. Below pH 6, dimerization affects our results and a quantitative analysis cannot be performed. However, we present evidence indicating that dimerization can occur only with the active conformation.

Experimental Section

Proteins. Salt-free, three-times crystallized α- (mol wt 25 000) and δ-chymotrypsin were obtained from either Worthington Biochemical Corp. (α-chymotrypsin, type CDI, lot numbers 3AF, 34S895, 35H793, 35A970, 35A912, and 35P692; δ-chymotrypsin, type CDD, lot number OGA) or Miles Laboratories, Inc. (lot number 1695C). Salt-free, five-times crystallized chymotrypsinogen A was obtained from Worthington Biochemical Corp. (type CGC, lot number 35E636). α-Chymotrypsin and chymotrypsinogen were purified by previously published methods (Nakagawa and Bender, 1970) and gave active-site titration values of 88–100% when determined by standard methods (Schonbaum et al., 1961). δ-Chymotrypsin was used unpurified and was 85% active by active-site titration. The results reported here are independent of protein source or purification procedure. Phenylmethanesulfonyl-α-chymotrypsin was prepared by adding 0.75 mL

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[1] Not all investigators agree with this interpretation (Blair et al., 1971; Marini and Martin, 1968, 1971); however, a satisfactory alternative explanation of the data has not been proposed. The great majority of results support this description.

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of a 0.075 M phenylmethanesulfonyl fluoride solution to a 1% solution of α-chymotrypsin buffered at pH 7.8 (Fahrney and Gold, 1963). Activity assays with *N*-acetyl-L-tryptophan ethyl ester indicated the protein was fully inactivated after 5 min.

All protein samples were lyophilized from 10^{-3} M HCl and stored at −50 °C until needed. Fresh enzyme stock solutions were prepared each day and were kept at about pH 3 before use to minimize autolysis. Protein concentrations were determined spectrophotometrically at 280 nm, using an extinction coefficient of 5.0×10^4 M^{-1} cm^{-1} (Dixon and Neurath, 1957).

Reagents. Proflavin hemisulfate, purchased from K & K Laboratories, was recrystallized in the dark from methanol and water three times, vacuum dried, and stored in the dark. Proflavin stock solutions were prepared fresh at least once a week and stored in the dark at 4 °C. Concentrations were determined spectrophotometrically at 444 nm using an extinction coefficient of 3.34×10^4 M^{-1} cm^{-1} (Bernhard et al., 1966). As solutions of proflavin deviate from Beer's law above 1.2×10^{-4} M (Glazer, 1965), care was taken to avoid final concentrations above 1.0×10^{-4} M throughout the work.

N-Acetyl-L-tryptophan *p*-nitrophenyl ester was obtained from Cyclo Chemical Corp. Other reagents were certified ACS grade or better. Buffer solutions, with ionic strength maintained by the addition of salt (usually KCl), were prepared according to Perrin (1974). Acetate (pH 3–5.5), phosphate (pH 6–8, 10.5–11), and borate (pH 8–10.5) were used as buffers. Normally, low buffer concentrations (0.005 M) were used; however, increasing buffer concentration to 0.1 M while maintaining constant ionic strength produced no measurable change in results.

Instrumentation. The stopped-flow apparatus used was a "home-built" device which has been described previously (Stoesz, 1977). The dead time of the instrument, calibrated using the reaction of H_2O_2 with dichromate in nitric acid, varied from 5 to 10 ms depending on the pressure used to drive the driving syringes. All transient amplitudes were corrected for this effect. Data were obtained with the aid of a Digital Equipment Corp. PDP 11/10 minicomputer and were fit to appropriate functional forms by weighted least-squares methods (Bevington, 1969). Most transients were first-order and absorbance readings spanning 2 to 3 half-lives with infinity absorbance values determined at 10 to 12 half-lives were used in analysis.

pH values were measured to ±0.02 unit at the temperature of the experiment.

Methods. The fraction of α-chymotrypsin present in the active conformation was determined by a method developed and described by Fersht and Requena (1971; Fersht, 1972). α-Chymotrypsin (4.0×10^{-5} M), incubated in a dilute buffer (0.005 M buffer, pH and salt concentration as desired), was mixed with an equal volume of a proflavin solution (1.8×10^{-4} M, 0.1 M phosphate buffer, pH 7.0) in the stopped-flow apparatus. A rapid burst of enzyme–proflavin complex formation was observed by monitoring the absorbance at 470 nm. The burst was followed by a slow, exponential increase in complex formation ($k_{obsd} = 2.9$ s^{-1}) due to the interconversion of inactive to active enzyme. The ratio of the amplitude of the initial burst (ΔA_0) to the total absorbance change (ΔA_∞) approximates the fraction of enzyme present in the active conformation in the initial α-chymotrypsin solution. Four to six determinations were done at each pH and ionic strength. ΔA_0 and ΔA_∞ could generally be determined to better than 0.5%.

The exact relationship between the absorbance changes and the ratio of active enzyme to total enzyme, $[E_A]/[E_T]$, is given by eq 1 (Fersht, 1972):

$$\frac{[E_A]}{[E_T]} = \frac{\Delta A_0}{\Delta A_\infty}\left\{\frac{\{([E_A]/[E_T])' + [F_0]/K_F'\}[F_\infty]}{(1 + [F_\infty]/K_F')[F_0]}\right\} \quad (1)$$

where K_F' is the experimentally determined proflavin dissociation constant, $[F_0]$ is the free proflavin concentration after the initial rapid burst of proflavin–enzyme complex formation but before the slow inactive to active interconversion can take place, $[F_\infty]$ is the free proflavin concentration at equilibrium and $([E_A]/[E_T])'$ represents the ratio of active to total enzyme at the final conditions of the reaction. We emphasize that eq 1 gives $[E_A]/[E_T]$ at the conditions of the α-chymotrypsin solution before mixing with the proflavin. If conditions before and after mixing are the same, the equation may be rearranged to give an exact solution for $[E_A]/[E_T]$.

The term in parentheses in eq 1 approaches 1.0 as the final proflavin concentration gets large. At the proflavin concentrations used here (ca. 9.0×10^{-5} M), the term is near 1.0 but was calculated exactly from supporting experiments. The required proflavin dissociation constant, K_F', was determined to be $3.6 \pm 0.5 \times 10^{-5}$ M at the final conditions of all the 0.2 ionic strength experiments (0.053 M phosphate, 0.1 M KCl, pH 7.0, 25 °C) with well-known spectrophotometric techniques (Bernhard et al., 1966). This value agrees with most literature values (Bernhard at el., 1966; Marini and Caplow, 1971; Taylor et al., 1973). The result was not dependent on salt concentration between 0.01 and 0.5 M KCl, to within the error limits given, and was used in calculations at all ionic strengths. $[F_0]$ and $[F_\infty]$ were determined from the proflavin dissociation constant, the total proflavin concentration, and the total enzyme concentration. Despite the numerous calculations required to determine the term in parentheses, the propagated error of the term makes only a small contribution to the error of the ratio of active to total enzyme ($\sigma < 0.005$).

An alternate derivation, using the change in extinction coefficient at 470 nm upon proflavin–enzyme complex formation, $\Delta\epsilon$, in place of K_F', yields eq 2:

$$\frac{[E_A]}{[E_T]} = \frac{\Delta A_0}{[E_T]} \times \left\{\frac{1}{\Delta\epsilon} + \frac{\left([E_T] - \frac{\Delta A_\infty}{\Delta\epsilon}\right)\left([F_T] - \frac{\Delta A_\infty}{\Delta\epsilon}\right)([E_A]/[E_T])'}{\Delta A_\infty\left([F_T] - \frac{\Delta A_0}{\Delta\epsilon}\right)}\right\} \quad (2)$$

where $[F_T]$ is the total proflavin concentration, $[E_T]$ is the total enzyme concentration, and the other symbols have already been described. Equation 2 enabled the calculation of $[E_A]/[E_T]$, where proflavin dissociation constants were not known. $\Delta\epsilon$ was determined in the stopped-flow apparatus using solutions with a known amount of enzyme–proflavin complex formation.

Nonspecific binding between α-chymotrypsin and proflavin makes a small contribution to the initial burst of complex formation, ΔA_0. This contribution was found to be 5.5 ± 1.0% of ΔA_∞ in a control experiment using either chymotrypsinogen or phenylmethanesulfonyl-α-chymotrypsin as a replacement for α-chymotrypsin.

A rapid activity assay (Fersht, 1972) was used to confirm the results of the proflavin assays at high pH. A stock solution of *N*-acetyl-L-tryptophan *p*-nitrophenyl ester in acetonitrile was diluted to a concentration of 1.0×10^{-4} M with 0.2 M sodium acetate at pH 4.5 and immediately mixed in the stopped-flow apparatus with an α-chymotrypsin solution in dilute buffer. The rate of *p*-nitrophenol release was monitored

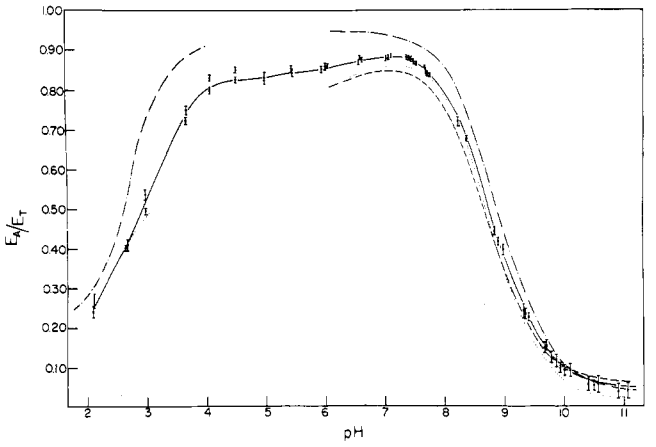


FIGURE 1: pH dependence of the fraction of α-chymotrypsin present in the active conformation at 25 °C as determined by the proflavin assay method. Conditions: Initial α-chymotrypsin solutions; 0.005 M buffer at ionic strengths 1.0, 0.2, and 0.05; 0.002 M buffer at ionic strength 0.01; acetate buffer from pH 3 to 6, phosphate buffer from pH 6 to 8 and pH 10.5 to 11, borate buffer from pH 8 to 10.5; ionic strength maintained with KCl; ca. 4.0×10^{-5} M α-chymotrypsin. Initial proflavin solutions; ca. 1.8×10^{-4} M proflavin, 0.1 M phosphate buffer, pH 7.0. The lines shown are computer fits to Scheme II above pH 6 but are hand drawn below pH 6. The points shown are for ionic strength 0.2. The data points at other ionic strengths were left off for clarity: (- • -) ionic strength 1.0; (—) ionic strength 0.2; (• • •) ionic strength 0.05; (- - -) ionic strength 0.01.

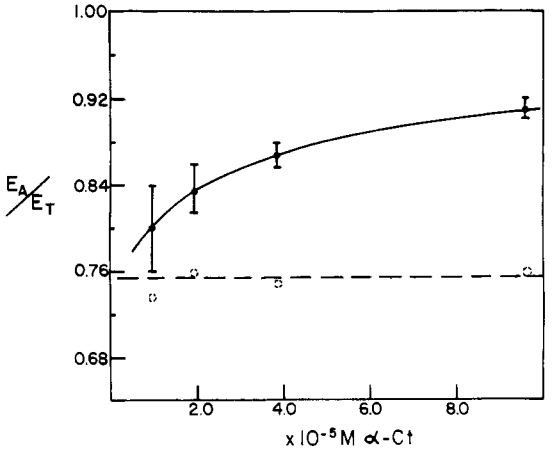


FIGURE 2: Concentration dependence of the fraction of α-chymotrypsin present in the active conformation at pH 4.45 and 25 °C. Conditions: Initial α-chymotrypsin solutions; 0.005 M acetate, ionic strength 0.2 maintained with KCl, pH 4.45 ± 0.05, α-chymotrypsin concentration as shown. Initial proflavin solutions as for Figure 1. Solid lines approximate the data and the dashed lines and points have been corrected for dimerization effects as described in the text.

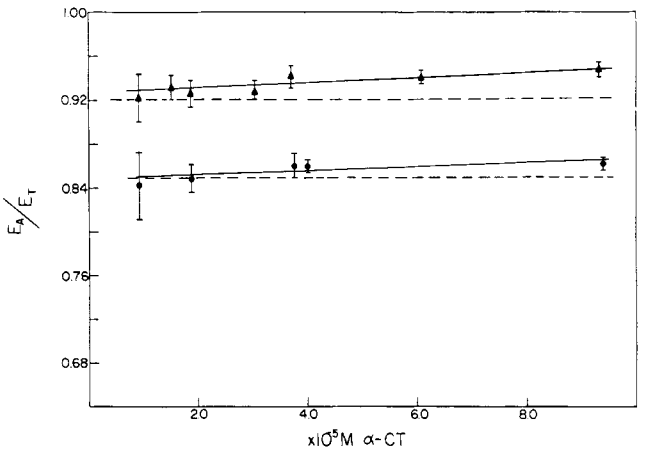


FIGURE 3: Concentration dependence of the fraction of α-chymotrypsin present in the active conformation at pH 6.0 and 25 °C. Conditions: Initial α-chymotrypsin solutions; 0.005 M phosphate, pH 6.0 ± 0.05, (●) ionic strength 0.2 (KCl), (▲) ionic strength 1.0 (KCl), α-chymotrypsin concentrations as shown. Initial proflavin solution as for Figure 1. The solid lines approximate the data and the dashed lines have been corrected for dimerization effects as described in the text.

at 340 nm from 9.1 to 19.0 ms (including the instrument dead time). A calibration curve of initial rates vs. active enzyme concentration was constructed using α-chymotrypsin incubated at pH 7.0 and used to determine the concentration of active enzyme present in a 1.9×10^{-4} M α-chymotrypsin solution at pH 10.8. This result was corrected for the small conversion of inactive to active enzyme which occurred at pH 4.5 during the dead time of the instrument and the observation time.

Results

The pH and ionic strength dependencies of the active to inactive conformational transition at 25 °C, evaluated using eq 1 and reported as the fraction of active enzyme present, $[E_A]/[E_T]$, are shown in Figure 1. The data are in general agreement with previous determinations at ionic strength 0.1 (Fersht and Requena, 1971). Errors are reported as standard deviations propagated by well-known methods from the original data (ΔA_0, ΔA_∞, K_F', etc.). Single first-order transients were obtained when the initial pH of the α-chymotrypsin solution was between pH 6 and 11. Between pH 2 and 6, two transients were observed; the usual inactive to active isomerization with the expected rate was preceded by a second, faster process, which also represents an increase in proflavin binding. The values of the fraction-active in this pH range were calculated using data collected after the fast process was 90 to 95% complete; therefore, the amplitude of the fast process has been included in the initial burst (ΔA_0). This rapid process has a maximum amplitude at pH 4 and, although quite small at low ionic strength (0.05 M), increases with increasing ionic strength. These pH and ionic strength dependencies are similar to those observed for dimerization (Aune and Timasheff, 1971; Aune et al., 1971); therefore, we believe we are observing dissociation of dimers upon dilution of the protein, followed by the normal inactive to active isomerization. Similar observations have been reported for concentrated α-chymotrypsin solutions at pH 3.89 by Gilleland and Bender (1976), who also attributed the fast process to dimerization.

The concentration dependencies of the fraction-active, $[E_A]/[E_T]$, at pH 4.45 and 6 and ionic strength 0.2 and 1.0 are reported in Figures 2 and 3. As concentration increases, the fraction-active increases because it contains contributions from both dimers and active conformer monomers. At pH 4.0 and ionic strength 1.0, the amplitude of the dimer-dissociation process is large and the amplitude of the normal inactive to active transition is too small to measure. Greater than 95% of the enzyme must be in either an active state or in a dimer, thus preventing accurate determination of $[E_A]/[E_T]$ at ionic strength 1.0 from about pH 3.0 to 6.0 (Figure 1).

Let us assume that both active and inactive isomers of α-chymotrypsin form dimers, in general with differing equilibrium constants (Scheme I). We can identify the slowest transient observed with the normal inactive to active transition (E_I

SCHEME I.

$$\begin{array}{ccc} E_{I2} & \overset{K'}{\rightleftharpoons} & E_{A2} \\ \Updownarrow K_{dim,I} & & \Updownarrow K_{dim,A} \\ 2E_I & \underset{\text{slow}}{\overset{K}{\rightleftharpoons}} & 2E_A \overset{+2F}{\underset{K_F}{\rightleftharpoons}} 2E_A{\cdot}F \end{array}$$

$\rightleftharpoons E_A$). The remaining relaxation could be due to any of the other three processes. It could only be the dissociation of inactive dimers ($E_{I2} \rightleftharpoons 2E_I$) if both the conformational isomerization in the dimeric state ($E_{I2} \rightleftharpoons E_{A2}$) and the dissociation of active dimers ($E_{A2} \rightleftharpoons 2E_A$) were fast. However, if this were the case the entire system would relax by the upper pathway and only one transient would be observed. The process could not be the inactive to active transition in the dimeric state ($E_{I2} \rightleftharpoons E_{A2}$), because initial rates of the process are observed to follow second-order kinetics (Gilleland and Bender, 1976). Therefore, we conclude that the fast relaxation is due to the dissociation of active conformation dimers. It is also true that either the dissociation of inactive dimers ($E_{I2} \rightleftharpoons 2E_I$) or the conformational isomerization in the dimeric state ($E_{I2} \rightleftharpoons E_{A2}$) must be slower than the monomeric inactive to active transition ($E_I \rightleftharpoons E_A$). If this were not the case, the system would relax as a single process. Two possibilities remain: (1) dissociation of inactive dimers ($E_{I2} \rightleftharpoons 2E_I$) is slow, in which case the measured fraction-active is:

$$([E_A]/[E_T])_{app} = \frac{2[E_{I2}] + 2[E_{A2}] + [E_A]}{[E_T]} \quad (3)$$

or (2) the inactive to active transition in the dimer is slow, in which case we obtain eq 4 for $[E_A]/[E_T]$.

$$([E_A]/[E_T])_{app} = \frac{2[E_{A2}] + [E_A]}{[E_T]} \quad (4)$$

We favor the second case because we would expect the conformational transition in the dimer to be no faster than the process in the monomer, if it can occur at all. Assuming eq 4 applies, the observation that $[E_A]/[E_T]$ approaches 1.0 under conditions of maximal dimerization (ionic strength 1.0, pH 4.0) indicates the active conformation forms dimers while the concentration of inactive dimers is negligible. Optical rotation (Parker, 1967) measurements as a function of salt concentration support this conclusion.

The apparent dimerization constant, $K_{dim,app}$, as reported in the literature, gives the ratio of dimer to monomer concentration. We have argued above that the concentration of inactive dimers is negligible; therefore, $K_{dim,app}$ is given by eq 5.

$$K_{dim,app} = \frac{[E_{A2}]}{[E_I] + [E_A]} \quad (5)$$

Equations 4 and 5, the extensive data on α-chymotrypsin dimerization of Aune and Timasheff (1971; Aune et al., 1971), and our data (Figures 1–3) can be combined to yield the true equilibrium constants, K and $K_{dim,A}$ of Scheme I. Values of $[E_A]/[E_T]$, corrected for dimer formation, are shown by dashed lines in Figures 2 and 3. Several approximations, such as assuming that the salt dependency at pH 6 is the same as that at pH 5.1, were required to extract values for the apparent dimerization constant from the literature, and the adjustments to our data must be regarded as qualitative. Our model adequately accounts for the concentration dependence of Figures 2 and 3, providing additional evidence that the inactive conformation forms no dimers under these conditions.

Above pH 6, the fraction-active values, $[E_A]/[E_T]$, show little concentration dependence, as predicted by the apparent self-association constants (Egan et al., 1957; Horbett and Teller, 1974; Rao and Kegeles, 1958; Pandit and Rao, 1975), and only one relaxation is observed in the stopped-flow proflavin assay. Thus, association problems are minimal. However, the fraction-active is still strongly dependent on the ionic strength between pH 6 and 8. Figure 4 shows the dependence of the fraction-active on ionic strength at pH 7, using

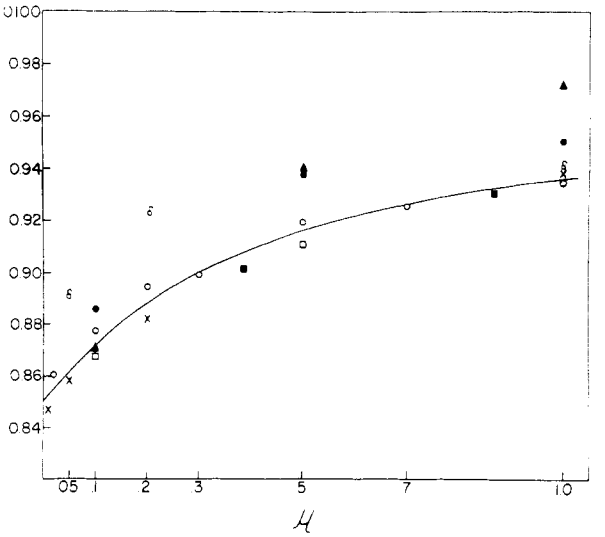


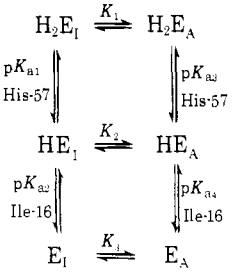
FIGURE 4: Ionic strength dependence of the fraction of α-chymotrypsin present in the active conformation at pH 7.0 and 25 °C. Conditions: Initial α-chymotrypsin solution; 0.005 M phosphate, pH 7.0 ± 0.05, α-chymotrypsin ca. 4.0×10^{-5} M, ionic strength maintained by addition of these salts: (⊙) NaCl, (×) KCl, (□) KSCN, (▲) KF, (●) $LiNO_3$, (■) $(NH_4)_2SO_4$, and (δ) δ-chymotrypsin with added KCl. Initial proflavin solution as for Figure 1. The solid line is drawn through the KCl and NaCl points.

a variety of salts covering the entire range of the lyotropic series (von Hippel and Schleich, 1969). Similar results were obtained at pH 6. The salt dependence of δ-chymotrypsin which does not dimerize (Neet and Brydon, 1970; Aune and Timasheff, 1971) is included to show that the effects are not due to association. In fact, the literature values for association would have to be wrong by more than an order of magnitude if they were to account for the results of Figure 4 (Aune and Timasheff, 1971). Experiments involving $LiNO_3$, KSCN, $(NH_4)_2SO_4$, and KF were analyzed using eq 2, because the proflavin dissociation constants are not known in these salts. Hence, the errors in these determinations are somewhat larger than the errors of the NaCl and KCl experiments. Considering the wide variety of salts used, it is very surprising that all affect the equilibrium in approximately the same manner.

We also observe that even at high pH, the active conformer possesses a measurable degree of stability (Figure 1). While errors in $[E_A]/[E_T]$ are large when $[E_A]/[E_T]$ is small, similar results were obtained at all the temperatures and ionic strengths we employed (Stoesz and Lumry, 1978), suggesting the generality of the conclusion. To further confirm our finding, we have used the rapid activity assay (Fersht, 1972), based on *N*-acetyl-L-tryptophan *p*-nitrophenyl ester hydrolysis as described in the Experimental Section. At pH 10.80 (25 °C, ionic strength 0.2), we find 8.0 ± 1.0% active conformation present, which, although slightly larger, agrees with the proflavin binding assay results to within the errors of both procedures.

The pH dependence of data obtained below pH 6 is affected by dimerization and several ionizations, so a complete analysis is difficult. Data obtained between pH 6 and 11 appear to reflect only two pK_as. At no pH in this range does the protein exist totally in either the active or inactive conformation. A mechanism, previously used for δ-chymotrypsin (Fersht, 1972), which can account for these observations is given in Scheme II. None of the six states can be neglected, as they all exist in appreciable concentrations at some pH. The pK_as of Ile-16 in the inactive and active states are pK_{a2} and pK_{a4}, respectively. The deprotonation of this group results in the large decrease

SCHEME II.



in fraction-active at high pH. We delay identification of pK_{a1} and pK_{a3} until later, but deprotonation of the responsible group results in the small increase in active conformation as pH is raised from 6 to 7. The equilibrium constants governing the ratio of active to inactive isomer at low, medium, and high pH are K_1, K_2, and K_3, respectively. The active species is most stable in the singly protonated state (HE_A) while the inactive species is more stable in the unprotonated state (E_I).

The pH-dependent apparent equilibrium constant controlling the active to inactive ratio is given by eq 6.

$$K_{app} = \frac{[E_A]_{all\ forms}}{[E_I]_{all\ forms}} = \frac{\left(K_2 + \frac{K_{a2}^{\cdot} K_3}{[H]} + \frac{K_1[H]}{K_{a1}}\right)}{\left(1 + \frac{K_{a2}}{[H]} + \frac{[H]}{K_{a1}}\right)} \qquad (6)$$

Data between pH 6 and 11 were fit to the mechanism as follows. First, points between pH 7.7 and 11 were fit to a single ionization curve with variable end points to determine approximate values of K_2, K_3, and pK_{a2}. These were used as starting values for a five-parameter weighted linearization of parameters least-squares fitting procedure using all the data (Bevington, 1969). The procedure worked well in general and the reduced chi-square values obtained were between 0.5 and 1.5, indicating a good fit of the data to the mechanism. Of course, even though errors in the experimental data are small, errors in the five fitting parameters are somewhat larger because of the large number of such parameters necessary to describe the equilibrium.

Equilibrium constants for Scheme II as a function of ionic strength at 25 °C are reported in Table I. Several things should be noted. First, K_1 and K_2, the conformational equilibrium constants for the doubly and singly protonated species, follow the salt dependence seen in Figure 4 for the observed data. No salt-dependent trends are noted for K_3, at least to within its rather large standard deviations. pK_{a1} and pK_{a2} show little salt dependence between 0.01 and 0.2 ionic strength. This result was unexpected. pK_{a3} and pK_{a4} can be calculated from the data; however, their propagated errors are too large to make their ionic strength dependence useful. At ionic strength 0.2, pK_{a3} is 6.67 ± 0.22 and pK_{a4} is 10.09 ± 0.32.

At ionic strength 1.0, only the pK_a due to Ile-16 is necessary to describe the data. This curve was fit to a single ionization with variable end points. There are two possible reasons for the disappearance of pK_{a1} and pK_{a3}. The pK_a could have shifted below pH 6, in which case it would be obscured by dimerization. Alternatively, K_1 may be approximately equal to K_2 at high ionic strength. We favor the latter explanation and have found that at low temperatures a very small pH dependence is observed in this region (Stoesz, 1977).

TABLE I: Ionic Strength Dependence of the Equilibrium Constants of Scheme II at 25 °C.[a]

ionic strength[b]	K_1	K_2	K_3	pK_{a1}	pK_{a2}
0.01	4.00	8.17	0.0597	7.10	7.78
	±0.21	±0.76	±0.0106	±0.10	±0.05
0.05	3.95	9.74	0.0203	7.06	7.78
	±0.33	±1.05	±0.0204	±0.10	±0.05
0.2	5.60	10.45	0.0490	6.94	7.76
	±0.37	±0.60	±0.0105	±0.10	±0.05
1.0		18.84	0.0345		7.64
		±3.74	±0.0104		±0.05

[a] Least-squares fit of the data from pH 6 to 11 in Figure 1 to Scheme II. [b] Maintained with KCl. [c] A one pK_a mechanism was used to fit this data, see text for details.

Discussion

The transition between the active and inactive forms of α-chymotrypsin can be observed in substrate binding, inhibitor binding, circular dichroism, optical rotation, ultraviolet spectroscopy, and fluorescence spectroscopy with varying degrees of precision. In a study such as ours, it is essential to use a method capable of yielding the highest quality data as rapidly as possible. We have chosen a stopped-flow proflavin binding assay, which was developed by Fersht and Requena (1971) and is capable of precise equilibrium measurements, and have combined it with on-line computer data acquisition for increased speed and precision. This paper reports use of the method to study the active–inactive equilibrium as a function of pH and salt concentration. Our findings agree qualitatively and semiquantitatively with previously reported data (Fersht and Requena, 1971), but there are two differences between our work and previous work: (1) We have detected an additional participating ionization process at neutral pHs. This ionization has a very small effect on the conformational equilibrium, but the effect is seen at all ionic strengths (except 1.0) and at all temperatures investigated (Stoesz and Lumry, 1978). A similar ionization has been reported to exist in δ-chymotrypsin (Fersht, 1972). (2) In addition, we found that even at high pH the active conformation retains a significant degree of stability; that is, about 5% of the α-chymotrypsin remains in the active conformation. While errors are large whenever the fraction of active enzyme approaches zero (Figure 1), a measurable amount of active α-chymotrypsin is observed at pH 11 for all ionic strengths and temperatures studied (Stoesz and Lumry, 1978). In fact, at 1 °C about 10% active conformer is present at pH 11. δ-Chymotrypsin has also been reported to have an appreciable amount (12.4%) of active conformer present at high pH (Fersht, 1972). Our high pH data are consistent with inhibitor binding results (Valenzuela and Bender, 1970) which show a 10- to 12-fold decrease in inhibitor binding to α-chymotrypsin at pH 10.0.

Salt Effects. Canady and co-workers have presented spectroscopic (circular dichroism, optical rotation) and thermodynamic (substrate and inhibitor binding) evidence for the existence of two salt-dependent forms of α-chymotrypsin near neutral pH (Cuppett et al., 1971; Cuppett and Canady, 1970; Royer et al., 1969, 1971). We observe a similar salt dependence (Figure 4) and have identified the two states as the active and inactive conformations. The salt effects are not a transition from one state to another, but rather a perturbation of the active–inactive equilibrium. The circular dichroism spectra spectra of active and inactive α-chymotrypsin (Stoesz, 1977) can be used with the fraction-active results (Figure 4) to calculate the expected circular dichroism salt dependency; good agreement with both our own and Canady's circular dichroism salt effects (Cuppett et al., 1971) is obtained.

Several explanations for the salt dependency of the fraction-active at pH 7 (Figure 4) and of the equilibrium constants,

K_1 and K_2 (Table I), are possible. There may be differences in hydration of the two states; however, the effects would be small and should follow the lyotropic series (von Hippel and Schleich, 1969). They do not and we can rule out this possibility. The salt effects could be due to electrostatic interactions, but the absence of strong salt dependence at low ionic strengths refutes this hypothesis. Preferential binding of salt to the active conformation would also produce the observed effects. A very simple mechanism is one in which one salt-binding site on the protein is allowed to change affinity during the isomerization. The data fit this scheme well (evaluated with a K_{app} vs. log [salt] plot) and show a salt dissociation constant that changes from 0.50 M in the inactive state to 0.08 M in the active state. In spite of the good fit, the interpretation is not conclusive and other mechanisms, such as two binding sites, fit as well. The effects do appear to be due to changes in the amount of salt bound during the transition.

Above pH 9, ionic strength effects are reduced to a minimum; therefore, the salt-binding site (sites) must lose charge between pH 7 and 9. This observation suggests one site is either a histidine or an α-amino group and the anion is the important salt species.

Ionization of the α-amino group of Ile-16 gives rise to pK_{a2} (see Scheme II), but the origin of pK_{a1} is not known. Its pK_a identifies it as a histidine (His-40, -57) or an α-amino group (Ala-149, Cys-1). δ-Chymotrypsin, which has no free N-terminal Ala-149, shows a similar pK_a, so we can exclude this group (Fersht, 1972). Results obtained with *N*-Me-His-57-α-chymotrypsin show no pK_{a1} (Stoesz, 1977); therefore, we will tentatively assign the ionization to His-57. It is interesting that this pK_a is different in the active and inactive forms of the enzyme, thus indicating there is a linkage between the ion pair and the charge-relay system; the linkage is weak (0.37 kcal at 25 °C) and probably has a minor role in the catalytic mechanism. Interestingly, the pK_a of His-57 in chymotrypsinogen also decreases about 0.5 pH unit upon activation to α-chymotrypsin (Cruickshank and Kaplan, 1974), thus providing additional evidence that the conformational changes which accompany the activation process are similar in some respects to the inactive to active interconversion.

The lack of ionic strength dependence, within error, of pK_{a1} and pK_{a2} is puzzling (Table I). One would predict that the measured pK_a of an imidazole or α-amino group should increase significantly with increasing ionic strength. The opposite is observed but the large errors, especially in pK_{a1}, prevent a definitive statement. Existing theories, such as the Debye-Huckel treatment, are based on a charged, hard-sphere model in very dilute solution (ionic strength <0.001), which are conditions not realized for a charged group closely attached to a protein. Other complications, including incomplete exposure of the groups to solvent and interactions with other charged groups of the protein, are certainly important.

High-pH Behavior. At pH 11, Ile-16 is deprotonated even inside the enzyme; thus, our results show the active conformation can exist without the ion-pair interaction. The difference in the free energy of the transition with Ile-16 protonated (K_2) and deprotonated (K_3) is 3.2 kcal and this can be viewed as the free energy of stabilization due to the ion pair. It should not be viewed as the free energy of the ion pair because the two groups may undergo small changes in orientation to form new interactions in form E_A. These changes may be similar to those observed in the crystal when Ile-16 is deprotonated in one molecule of the asymmetrical dimer (Mavridis et al., 1974). Deprotonated Ile-16 moves toward Ser-190, and its α-amino group may form a hydrogen bond with the serine side chain. Asp-194 rotates toward the amido group of Trp-141 with which it may hydrogen bond. Both groups remain inside the protein, and no large changes are observed in the substrate-binding region; therefore, a priori we would classify this new conformation as an active species. While crystal-packing restrictions will affect these results and what occurs in solution may be different, the most important point is that new interactions are formed within the active conformation when the salt bridge is broken.

Dimerization Behavior. Dimerization of the inactive conformation of α-chymotrypsin is not observed, indicating there are changes in the dimer interface region during the conformational transition. Inactive conformation dimers are at least one order of magnitude less stable than active conformation dimers. This corresponds to only about 1.4 kcal of free energy difference and could result from a small rearrangement of strongly interacting groups or the large movement of weakly interacting groups. Dimerization, as observed in the crystal structure, is accompanied by many structurally asymmetrical changes in the interface region (Tulinsky et al., 1973), with the main chain from Trp-215 to Ser-218 and residues Phe-39 and Met-192 showing especially large (>2.5 Å) movements. These and other small changes throughout the molecule suggest there are no rigid spatial requirements for dimer formation, but that the molecules are able to accommodate each other to reduce bad interactions and strengthen good interactions with considerable versatility. Charge-charge interactions, of which the His-57 to Tyr-146 C-terminal and Asp-64 to Ala-149 N-terminal interactions are thought to be most important, account for the pH dependence of the free energy of association (Aune and Timasheff, 1971). His-57 is not observed to change position in any of the pH conformers studied by Tulinsky and co-workers (Tulinsky et al., 1973; Mavridis et al., 1974; Vandlen and Tulinsky, 1973) and can be excluded on this basis. Asp-64 and Ala-149 show no large movements when the Ile-16 to Asp-194 ion pair is broken in the crystal, but Tyr-146 is claimed to move about 1.5 Å (Mavridis et al., 1974), suggesting that this group may be responsible for the destabilization of inactive dimers. The applicability of the above result to the situation in solution is uncertain at best. The most promising hypothesis is that movement of a group involved in a charge-charge interaction (probably the C-terminal Tyr-146) during the active to inactive transition is the cause of the decrease in stability of inactive state dimers.

The results of this paper make it possible to move to the more profound level of investigation possible with temperature and pressure as major variables. In the next paper, we report characteristics of the behavior of chymotrypsin revealed by temperature studies.

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