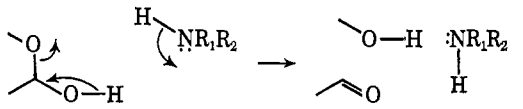
that is consistent with a steric interpretation. Primary and secondary amines do not appear to act as simultaneous proton donor–acceptors.



Experimental evidence that points to such an interpretation can be equally well explained on the basis of steric effects.

The experimental data thus strongly suggest that pyridine–phenol, pyridine–4-nitrophenol, diethylamine–phenol, and other acid–base mixtures function as general base catalysts through the action of the conjugate acid–base ion pairs. Owing to traditional difficulties in distinguishing between different mechanisms that contain identical transition states except for the distribution of atoms, it is very difficult to unequivocally disprove the existence of a concerted general acid–base mechanism in the mutarotation reaction. All of the experimental data reported to date are consistent with the interpretation that there is no such mechanism.[1,2,4–6]

Acknowledgments. The authors gratefully acknowledge numerous stimulating discussions with Professor Jack Halpern and Professor Richard L. Schowen.

Studies of the Chymotrypsinogen Family of Proteins. VI. Characterization of the Conformational Variation of Chymotrypsin[1]

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Abstract: The structural variation of α-chymotrypsin has been studied in the acid pH region and is shown to consist of two distinct equilibrium situations. The first of these, transition $A_a \rightarrow A_b$, is a thermodynamically small structural change and is described by changes in the optical rotatory dispersion. The second, transition I, is a thermodynamically large conformational change which is uniquely described by changes in the ultraviolet absorption spectrum. It is shown that transition I at pH 2.0 is a strongly cooperative, two-state transition and that the thermodynamic behavior is consistent with the Brandts model of protein unfolding.

Considerable recent effort has been focused on the thermodynamic changes occurring during polypeptide and protein conformational transitions as a means for obtaining information about the globular protein in solution and an understanding of the thermodynamic determination of the conformation. Of particular significance is the work of Brandts who has been successful in describing the thermally induced unfolding of chymotrypsinogen A[2,3] and ribonuclease[4] in terms of a relatively simple model assuming that only two unique thermodynamic states of the protein exist under experimental conditions. We now wish to apply this analysis to chymotrypsin and a variety of its chemical derivatives for the purposes of testing the method of Brandts and extending it as a phenomenological tool to aid in the understanding of protein thermodynamic stability. In addition, this type of analysis of the chymotrypsinogen family is necessary for any fundamental understanding of chymotryptic catalysis.

The application of the Brandts method of analysis requires that the conformational transition under consideration be well approximated by simple equilibrium between two distinct thermodynamic states. This is necessary in order to obtain meaningful thermodynamic quantities from the experimental data. Although Brandts was able to demonstrate two-state behavior in the unfolding transition of chymotrypsinogen in a straightforward manner using ultraviolet absorbance, the situation for chymotrypsin is more complicated, as is indicated by an apparent discrepancy between the optical rotatory dispersion (ORD) and ultraviolet absorption changes for the thermal transition at pH 2.0.[5] Therefore, before considering in detail the thermodynamics of the thermal transition of chymotrypsin, it is necessary to describe the conformational states of this protein in the acid to neutral pH region.

A clue to the source of difficulty in chymotrypsin was provided by the observation by Rupley, Dreyer, and Neurath[6] that this protein exhibits a marked variation in optical activity with pH. Subsequently, it was shown that these changes were due primarily to changes in a single Cotton effect centered near 207 nm.[7] Parker attributed this change to the rupture of an ion pair stable only in the pH region from 3 to 9.[8] Such an ion pair in

(1) This is paper no. 39 from this laboratory. Please request reprint by this number. The work in this paper is from the Ph.D. Dissertation of R. Biltonen, University of Minnesota, 1965.
(2) J. Brandts, *J. Am. Chem. Soc.*, **86**, 4291 (1964).
(3) J. Brandts, *ibid.*, **86**, 4302 (1964).
(4) J. Brandts, *ibid.*, **87**, 2759 (1965); J. Brandts and L. Hunt, *ibid.*, **89**, 4826 (1967).
(5) B. Havsteen, B. Labouesse, and G. P. Hess, *ibid.*, **85**, 796 (1963).
(6) J. Rupley, W. Dreyer, and H. Neurath, *Biochem. Biophys. Acta*, **18**, 162 (1955).
(7) R. Biltonen, R. Lumry, V. Madison, and H. Parker, *Proc. Natl. Acad. Sci., U. S.*, **54**, 1018 (1965).
(8) H. Parker, Dissertation, University of Minnesota, 1967.

α-chymotrypsin has been reported by Matthews, *et al.*[9] It consists of the carboxylate of Asp-194 and the α-ammonium group of Ile-16, and Parker's explanation is qualitatively consistent with this description. Williams and coworkers[10] found no change in the solvent-perturbation spectrum of the protein over the pH range 2 to 7, a result also suggesting that the conformational changes may be small, but no other information bearing on this problem is available and the actual description of the change remains to be provided.

A somewhat similar confusion has existed with respect to the nature of the transition produced by heating α-chymotrypsin solutions in the acid pH region. The temperature dependence of the changes in 270–300-nm difference spectrum[5] and in the value of the optical rotation[5,11-13] have suggested a major conformation change of the protein, but these two observables did not provide the same quantitative values for the apparent thermodynamic changes in the transition. This discrepancy could mean that the thermal transition is not a two-state transition or that the temperature dependence of the absorbance differences or the optical-rotatory strength has not been properly taken into account. In view of the existence of the optical-rotation change with pH in the low-temperature state, it has appeared probable that this change is responsible for the discrepancy between the results calculated from the data obtained by the two methods. We have examined this possibility using the tentative scheme $A_b \rightleftarrows A_a \rightleftarrows B$. Following the labeling procedure previously established[7,14] the general low-temperature state is called A. Substate A_b is the stable form of state A at neutral pH; substate A_a is the stable form at acid pH. These substates differ in the ORD pattern, A_b having well-developed Cotton effects in the 202–207- and 224–228-nm[15] spectral regions superimposed on an invarient α-helix pattern. Substate A_a has much smaller effects at these wavelengths superimposed on the same α-helix ORD pattern. At pH 2 substate A_b is still not completely depopulated and at still lower pH values the unfolded state, state B, becomes favored even at low temperatures. The intersubstate transition is labeled $A_a \rightarrow A_b$. The reversible thermal unfolding process $A \rightarrow B$ is called transition I.

In this paper we will show that transition I can be uniquely separated from transition $A_a \rightarrow A_b$, and that the enthalpy and entropy differences between states A_a and A_b are small compared to those between state A and state B. This allows us to describe transition I as a transition between a single low-temperature state, state A, and the high-temperature state, state B, and it will be shown that this transition is adequately approximated as a two-state transition, *i.e.* an equilibrium between two and only two macroscopic states.

(9) B. Matthews, P. Sigler, R. Henderson, and D. Blow, *Nature*, **214**, 652 (1967).
(10) E. J. Williams, T. T. Herskovits, and M. Laskowski, Jr., *J. Biol. Chem.*, **240**, 3574 (1965).
(11) J. Schellman, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **30**, 450 (1956).
(12) J. Brandts, Dissertation, University of Minnesota, 1961.
(13) J. Brandts and R. Lumry, *J. Am. Chem. Soc.*, **83**, 4290 (1961).
(14) R. Biltonen, R. Lumry, V. Madison, and H. Parker, *Proc. Natl. Acad. Sci. U. S.*, **54**, 1412 (1965).
(15) ORD studies by Cane (W. Cane, Dissertation, University of Minnesota, 1967) and unpublished circular dichroism observations by M. Lund and R. Lumry show that the Cotton effect in the 224–228-nm region is largest in α-chymotrypsin at neutral pH values.

Experimental Section

Materials. α-Chymotrypsin (three times crystallized activation product of three times crystallized chymotrypsinogen A, dialyzed salt-free and lyophilized) was obtained from Worthington Biochemical Corp., Freehold, N. J. Chromotography[16] of the sample according to the method of Hirs[17] at 4° indicated it to be homogeneous. As a result, the material was used without further purification. All chemicals were of reagent grade and used without further purification.[18]

Preparation of Protein Solutions. All solutions were prepared with deionized water using HCl to adjust the pH and KCl to adjust the ionic strength. For all temperature studies the deionized water was boiled prior to use to effect removal of dissolved gases so that bubbles would not form on heating. The dry protein was added slowly with constant stirring to the solvent so that neither a cloudy nor foaming solution was produced.

For studies which required a single solution per experiment, the dry protein was dissolved in water at the desired pH and ionic strength. Prior to use the solution was filtered through a 25-μ sintered-glass filter to remove any insoluble matter. In other experiments the dry protein was dissolved in deionized water to a concentration greater than desired in the final experiment. This stock solution was then filtered and diluted with water at the appropriate pH and ionic strength to provide a final solution of the desired composition.

The concentrations of all protein solutions was determined after filtration and dilution by measurement of the optical density at 280 nm using a specific extinction coefficient of 2.00 l./(g cm).

Solubility Measurements. The concentration of state B can be directly measured from the loss of solubility in a buffer containing high salt concentration. The procedure used has been described by Eisenberg and Schwert.[19]

Difference Spectrum Measurements. All difference-spectrum measurements were made using a Cary, Model 15, recording spectrophotometer. The protein solutions in both the sample and reference compartments were made from the same stock solution. The optical density of the solution was between 1.0 and 1.5 providing a difference of approximately 0.1 to 0.15 optical-density unit between high- and low-temperature forms of the protein.

Both sample and reference solutions were kept at constant temperature by use of thermostated cell holders. The temperature of the sample solution was determined by direct immersion of a calibrated thermistor into the solution. The temperature could be determined with an accuracy of 0.02° and was constant within this limit after a 10-min equilibration time at all temperatures. The reference solution was covered with a standard glass cap. The sample solution was covered with an air-tight Teflon cap through which the thermistor was inserted. Evaporative losses were noted only when the Teflon cap was improperly placed, and the solution was allowed to sit at high temperatures for several hours.

Optical Rotatory Dispersion Measurements. ORD measurements were made with a Cary 60 recording spectropolarimeter using a 5-cm fused-silica cell. The solution was maintained at constant temperature with a thermostated cell holder. The temperature was measured in the manner described above. The cell was checked for any temperature sensitivity but none was found over the temperature range 15 to 60°. All measurements were made with a slit width of 0.1 mm.

Results

Thermodynamic Calculations. All calculations of thermodynamic quantities given were made assuming a two-state equilibrium. The equilibrium constant for such a transition between state A and state B is

$$K = (\epsilon - \epsilon_A)/(\epsilon_B - \epsilon) \qquad (1)$$

where ϵ_A, ϵ_B, and ϵ are values for any observable for

(16) R. Biltonen, Dissertation, University of Minnesota, 1965.
(17) C. H. W. Hirs, *J. Am. Chem. Soc.*, 77, 5743 (1955).
(18) It has been found by Yapel, *et al.* (A. Yapel, R. Lumry, M. Han, A. Rosenberg, and D. Shiao, *ibid.*, **88**, 2573 (1966)) that commercial preparations of α-chymotrypsin contain varying amounts of small-molecular-weight contaminants. These, however, had no apparent effect on transition I since no quantitative differences in the transition were observed with different preparations.
(19) M. A. Eisenberg and G. W. Schwert, *J. Gen. Physiol.*, **34**, 583 (1951).

state A, state B, and the system, respectively. The standard free energy change is

$$\Delta \bar{F}^\circ = -RT \ln K \quad (2)$$

and the standard enthalpy change can be calculated from the van't Hoff relation at any temperature by

$$\Delta \bar{H}^\circ = -R[\partial \ln K/\partial(1/T)]_p \quad (3)$$

Reversibility. If meaningful thermodynamic quantities are to be calculated from the experimental data, it must be established that a true equilibrium exists, *i.e.*, the transition is completely reversible. The criteria for reversibility for protein systems are, on occasion, difficult to establish. This is particularly true when aggregation and autolysis of the protein can occur. The latter complications are particularly sensitive to concentration, ionic strength, and temperature, and it is important, therefore, to clearly delineate the exact conditions under which reversibility can be achieved.

The reversibility of transition $A_a \rightarrow A_b$ for chymotrypsin at high and low salt concentrations has been established elsewhere.[8] We have also verified the reversibility of this transition under our particular set of experimental conditions.

At pH 2 and with no added salt complete reversibility was observed when transition I was measured using loss of high salt solubility and changes in ORD and ultraviolet absorption during transition I. At other pH values reversibility of the changes in optical rotatory dispersion or loss of high salt solubility was not complete, although it was generally found that the changes in the ultraviolet absorption were reversible. At pH values below pH 2.0, where the chloride ion concentration was necessarily higher than 0.01 *M*, total reversibility was not obtained because of aggregation of the state B species. The latter fact was established from a close correlation between the degree of irreversibility and the fraction of heavy component ($s_{20,w} \approx 20$) in the sedimentation pattern of the protein solution on cooling. At pH values higher than 2.0 the absence of complete reversibility was the result of both aggregation and autolysis. The conclusion that autolysis was important was verified by comparison of the extent of irreversibility of the solution as a function of time at high temperatures. This behavior was determined in experiments performed at 48°, pH 3.0, and it was found that complete irreversibility was achieved in approximately 60 min with a 0.5% (by weight) protein solution.

We were able to achieve complete reversibility from highest experimental temperatures only in the ultraviolet spectra because these experiments required solutions only one-fifth as concentrated as the high salt solubility and the ORD experiments. Since both aggregation and autolysis reactions can be as high as second order in protein concentration, the rates of these processes were reduced by a factor of as much as 25 in the difference-spectrum experiments. The fact that we observed total reversibility with all three observables at pH 2.0 was a fortuitous coincidence of optimum conditions: at low pH aggregation was promoted primarily by higher chloride ion concentration whereas at higher pH aggregation was promoted by higher temperature[20] and autolysis was promoted by both higher temperature[16] and pH. Because we were unable to achieve complete reversibility at all pH values, comparison of the changes using all three observables could be made for transition I only at pH 2.0. Since we were able to achieve reversibility of the ultraviolet spectral changes for transition I at all pH values, and since we were able to identify the source of irreversibility of the other observables, we conclude that transition I is intrinsically reversible. Thermodynamic data for this transition with CT are given in the subsequent paper.[21]

(20) It will be shown in the subsequent paper[21] that the transition temperature for transition I is directly proportional to pH. Thus at pH values higher than 2, the temperature is an increasingly important source of irreversibility.

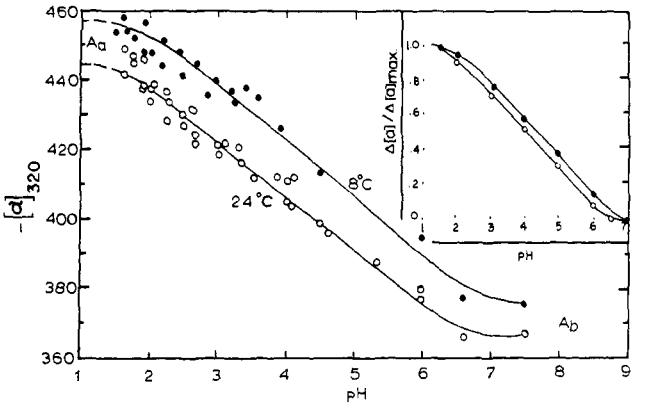

Figure 1. Specific rotation at 320 nm *vs.* pH for α-chymotrypsin at 0.01 *M* (Cl^-). Insert: Fractional change of $[\alpha]_{320}$ as function of pH. Consult text for details.

Transition $A_a \rightarrow A_b$. Parker[8,22] has described the ORD changes of chymotrypsin with pH at both high and low salt concentration at room temperature. We have repeated her experiments in 0.01 *M* chloride ion at 8 and 24°. The results are presented in Figure 1 as changes in the specific rotation at 320 nm, $[\alpha]$. A plateau in the pH dependence of $[\alpha]$ is seen at the neutral pH end of transition $A_a \rightarrow A_b$ at both temperatures. We assume that this value of $[\alpha] = [\alpha]_{A_b}$, the specific rotation of state A_b at the appropriate temperature. No such plateau was observed at the acid pH end of the transition, and hence we have no direct measure of the specific rotation of state A_a. Nevertheless, we can estimate $[\alpha]_{A_a}$ using two different sets of assumptions.

(1) The problem of analysis of transition $A_a \rightarrow A_b$ resides partially in the fact that at low pH (less than pH 2.5) chymotrypsin is partially in state B at all temperatures.[21] However, Marini and Wunsch[23] have shown that the protein is completely titrated at pH 2.0. If it is assumed that transition $A_a \rightarrow A_b$ as a function of pH mimics the titration behavior of the protein, then the transition will be essentially complete by pH 2.0, and $[\alpha]_{A_a}$ can be directly estimated by correcting for the presence of species B at pH 2.0 at the appropriate temperature to obtain the broken lines in Figure 1. It is now possible to estimate the apparent enthalpy change for transition $A_a \rightarrow A_b$, and we find that this quantity, $\Delta \bar{H}_A^\circ$, equals 4 kcal/mol and is independent of pH. It should be noted that even though transition $A_a \rightarrow A_b$ may be controlled by titration of the protein, it is not necessary that at pH 2.0 the protein is completely in state A_a at these temperatures; it is possible that at low pH the protein exists as an equilibrium mixture of states

(21) R. Biltonen and R. Lumry, *J. Am. Chem. Soc.* 91, 4256 (1969).
(22) H. Parker and R. Lumry, *ibid.*, 85, 483 (1962).
(23) M. A. Marini and C. Wunsch, *Biochemistry*, 2, 1459 (1963).

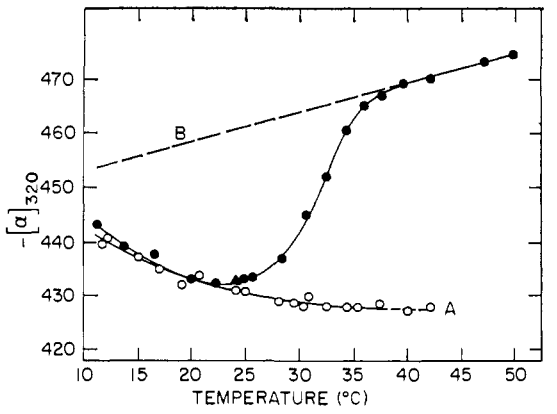

Figure 2. Temperature dependence of $[\alpha]_{320}$ of α-chymotrypsin, pH 2.0. Open circles are data at various pH values corrected for transition $A_a \rightarrow A_b$ and represent the correct temperature dependence of $[\alpha]_A$ at pH 2.0. Consult text for details.

A_a and A_b. If this is the case this estimate of ΔH_A° = 4 kcal/mol is a lower limit of the actual value of the standard enthalpy change for transition $A_a \rightarrow A_b$.

(2) If transition $A_a \rightarrow A_b$ is controlled by one or just a few of the ionizable groups of the protein, the above method for calculation of $[\alpha]_{A_a}$ may not be correct. We have previously[11] shown that transition I does not necessarily produce changes in the specific rotation *per se*, *i.e.*, $[\alpha]_{A_a} = [\alpha]_B$, the specific rotation in state B. (This assumption is probably not correct at all temperatures because the temperature dependence of $[\alpha]$ is a very strong function of conformational flexibility. Nevertheless, it allows another estimation of $\Delta\bar{H}_a^\circ$.) Thus, the observed changes may only reflect induced variation in relative population of species A_b due to shifts in the equilibrium toward state B. The temperature dependence of $[\alpha]_B$ extrapolated to lower temperatures is shown as line B in Figure 2. Using the value of $[\alpha]_B$ at the appropriate temperature for $[\alpha]_{A_a}$, we can calculate the relevant thermodynamic quantities and find $\Delta\bar{H}_A^\circ = +6$ kcal/mol which is independent of pH over the range from 2 to 5.

The error in the estimate of $\Delta\bar{H}_A^\circ$ using either set of assumptions is comparable in magnitude to $\Delta\bar{H}_A^\circ$ because of the imprecision of the data and the difficulty in establishing the exact base lines for states A_a and A_b. Nevertheless, this calculation provides the information that the standard enthalpy change for transition $A_a \rightarrow A_b$ is small. The thermodynamic result indicates that transition $A_a \rightarrow A_b$ is thermodynamically too small to be a conformational change in the usual sense of the term. This conclusion is supported by the fact that loss of high salt solubility, which is normally the result of extensive unfolding, was not observed during transition $A_a \rightarrow A_b$, and also by the fact that no change in the ultraviolet absorption spectrum could be correlated with the pH-dependent ORD change. Small changes in the ultraviolet absorption were detected below pH 5, but do not reflect changes in the local polarizability of the environment of the aromatic chromophores. Instead they are due to changes in the charge perturbations of solvent-exposed chromophores. This aspect of the ultraviolet properties of chymotrypsin will be discussed in more detail in the following paper.[21]

The absence of detectable differences between the two substates other than in their ORD patterns suggests that the ORD behavior is not due to large changes in conformation but rather is to be attributed to one or a few chromophores having unusually large rotational strength[21,24] or to many of the residue chromophores which experience small perturbations due to small but wide spread physical changes in amorphous regions of the protein.

Since transition $A_a \rightarrow A_b$ shows no significant change in ultraviolet absorption and no loss of high salt solubility, we can now monitor transition I by either loss of high salt solubility or changes in the ultraviolet absorption spectrum.

Transition I at pH 2.0. Lumry, *et al.*,[25] have recently discussed the two-state approximation for protein conformational transitions and proposed two tests for the validation of this approximation. First, if several different observables are used to follow the transition, yet all provide quantitatively identical values for the thermodynamic changes, then the transition is a two-state process. However, as mentioned previously, there appears in the literature an apparent discrepancy between the ultraviolet absorption changes and the optical rotatory dispersion changes for the thermally induced unfolding of chymotrypsin at pH 2. We have suggested that the reason for this discrepancy is the previous neglect of the temperature dependence of the specific rotation of chymotrypsin in the low-temperature state. However, before we begin a discussion of the temperature behavior of the optical rotation it is necessary to discuss other methods available to study thermal unfolding of proteins.

Schellman[11] has previously reported that the fraction of unfolded α-chymotrypsin can be directly measured as a function of temperature by loss of solubility in high salt buffer. Chymotrypsin is partially insoluble in high salt buffer at all temperatures at pH 2.0 although at pH 3.0, for example, at temperatures below 45° the protein is completely soluble in the high salt buffer. This result indicates that chymotrypsin is partially in the unfolded state at all temperatures at pH 2.0. For this reason state A will be operationally defined as that state which is completely soluble in high salt buffer and state B is that form of the protein completely insoluble in high salt buffer. The loss-of-solubility experiment was performed over a range of 15 to 45° for chymotrypsin at pH 2.0 and the results are given in Figure 3 in terms of fraction of protein in state B. As indicated on the graph chymotrypsin at 25°, for example, is approximately 7% in the unfolded state, state B.

The fraction of protein in state B as a function of temperature can also be determined from changes in the ultraviolet absorption spectra. The amount in state B was calculated assuming that the transition was of the two-state type and taking into account that at 25° α-chymotrypsin was 7% in state B. The results are also shown in Figure 3.

In Figure 2 the temperature dependence of the specific rotation at 320 nm for α-chymotrypsin is shown. The temperature dependence of the specific rotation for state A is indicated by the solid line labeled A. This line was established by measurement of

(24) R. Lumry and R. Biltonen in "Biological Macromolecules," Vol. 2, S. Timasheff and G. Fasman, Ed., Marcel Dekker, Inc., New York, N. Y., 1969, Chapter 2.

(25) R. Lumry, R. Biltonen, and J. Brandts, *Biopolymers*, 4, 917 (1966).

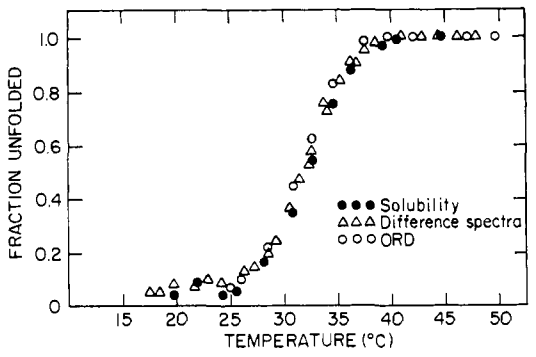

Figure 3. Fraction of α-chymotrypsin in state B as a function of temperature at pH 2.0.

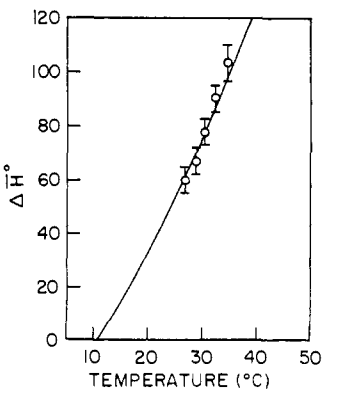

Figure 4. Temperature dependence of $\Delta H°$ of α-chymotrypsin at pH 2.0. Solid curve calculated using "best-fit" parameters for the Brandts free-energy function.[3]

the temperature dependence of the specific rotation of α-chymotrypsin at a number of pH values, and temperatures at which it is known that the protein does not undergo the thermal transition. It was adjusted for changes in optical rotation resulting from the temperature-induced changes in the equilibrium position of transition $A_a \rightarrow A_b$ to agree with the expected equilibrium position of transition $A_a \rightarrow A_b$ at pH 2.0. The latter adjustment was necessitated by the fact that changes in $[\alpha]$ as a function of temperature are not independent of pH. For example, at the midpoint of transition $A_a \rightarrow A_b$ a greater temperature dependence of $[\alpha]$ is to be expected because the equilibrium position at this pH will be the most temperature sensitive, whereas at pH 7, for example, no change in $[\alpha]$ due to a change in transition $A_a \rightarrow A_b$ will be observed. Thus base line A is the correct temperature dependence of $[\alpha]_A$ at pH 2.0. This line was also adjusted on the vertical scale to take into account that at 25° α-chymotrypsin is 7% in the state B form. The temperature dependence of the specific rotation in state B is indicated by line B which is an extrapolation of the specific rotation from above the transition region. Using lines A and B for the temperature dependence of specific rotations of states A and B the changes in $[\alpha]$ as a function of temperature can be used to calculate the fraction of proteins in state B as a function of temperature. The results of this calculation are shown in Figure 3.

As can be seen from the results in Figure 3, the fraction of protein unfolded as determined from the loss of high salt solubility, ultraviolet-spectral changes, or the optical-rotation changes is identical within small errors. This coincidence of changes in the different observables is one basis for the conclusion that the two-state approximation is valid for α-chymotrypsin at least at pH values below 3.5.

The apparent thermodynamic quantities for transition I of chymotrypsin at pH 2 can be calculated from the results in Figure 3 and are summarized in Table I along with the previous results of Havsteen, *et al.*[5] We see that our results using different methods show good agreement. Our calculated $\Delta H°$ values differ from the previously determined value obtained with ultraviolet spectral changes because α-chymotrypsin exists partially in state B at all temperatures at pH 2.0. If the results of Havsteen, *et al.*, were corrected for partial unfolding at all temperatures, their value of $\Delta\bar{H}°$ would be reduced by approximately 10% and would be in good agreement with our value.

A second test for the validity of the two-state approximation is based on the determination of the temperature dependence of the apparent enthalpy change for the transition as a function of temperature. If no relative extremum is observed in such a plot, then except under rare circumstances, the two-state approximation is valid. The result of such a calculation for transition I at pH 2.0 is shown in Figure 4. The apparent enthalpy change has been calculated from the tangents of the van't Hoff plot fitted to all the data. The solid line was calculated using the best-fit parameters for Brandts' free energy expression[3] and is discussed in the next paper of the series.[21] No obvious extremum in the apparent enthalpy change is observed, although the usual strong temperature dependence of the enthalpy change is observed. Taken by itself the second test is not particularly convincing since it is possible that the maximum could be obscured by a large and complex temperature dependence of the standard enthalpy change. Taken together with the first-test result and the similarity of the heat-capacity behavior between α-chymotrypsin[21] and chymotrypsinogen A,[2] the affirmative second test result adds confidence to the two-state interpretation of transition I for α-chymotrypsin.

Table I. Apparent Thermodynamic Quantities of the Thermal Denaturation of α-Chymotrypsin at pH 2.0

Observable	T_0, °C	$\Delta\bar{H}°$, kcal/mol	$\Delta S°$, eu
Solubility	32.1	82	268
Optical rotatory dispersion	31.6	84	275
Difference spectra	31.7	83	272
Difference spectra[a]	32.5	90	295

[a] Results from ref 5. These values of $\Delta H°$ and $\Delta S°$ were calculated without taking into account the fact that the protein was partially in state B at all temperatures at pH 2.0 resulting in values which are approximately 10% larger than the true values.

Discussion

We have shown that the structural changes of chymotrypsin in the acid pH region can be separated into two distinct equilibria. Transition $A_a \rightarrow A_b$ which presents itself only as a pH-dependent ORD change appears to be a thermodynamically small structural or ionization change of the molecule. The absence of changes in properties normally associated with conformational

changes strongly suggests that the apparent value of $\Delta\bar{H}_A°$ is a correct order of magnitude estimate. The change in the protein during transition $A_a \rightarrow A_b$ is not trivial. Parker and Lumry[26] have found that the protein always has the 202–207-mμ Cotton effect when it is functional. They found that this effect is apparently controlled by the ionization of a group with pK_a of about 3.0 at 0.1 *M* salt.[26] Kinetics studies of the reaction of diisopropylfluorophosphate with chymotrypsin carried out by Moon, Sturtevant, and Hess[27] showed that this reaction consists of two steps only the first of which induces changes in optical rotation. This step, which is presumably a reversible Michaelis–Menten complex formation, was found to have an activation energy of 4 kcal/mol, an interesting figure in light of our values for the enthalpy change of transition $A_a \rightarrow A_b$.

We have shown that transition I is a two-state transition by comparison of several different physical observables (test II of ref 25). Although this test is not rigorous, the fact that ORD, ultraviolet absorption, and solubility are different types of observables (gradual, stepwise, and all or none, respectively) strengthens our confidence in this test for this particular case. It must be realized, however, that the presence of another macroscopic state, *i.e.*, some state other than states A and B, would not have been detectable if the population was less than about 5%. Our present results only support the conclusion that the two-state approximation of transition I is valid within this error. Additional evidence for two-state behavior is provided by the observation of monotonically increasing values of the apparent enthalpy change for transition I as a function of temperature (test I of ref 25).

We have been unable to apply test II to transition I at pH values other than 2.0 because of our inability to obtain complete reversibility in the ORD and solubility experiments at higher pH values. However, test I can be applied using the changes in the ultraviolet absorption spectrum at other pH values since these changes are reversible. Our data at several other pH values satisfy test I.

The van't Hoff plot of data for transition I at pH 2.0 shows a very strong temperature dependence of the enthalpy change (Figure 4) which must be due to a heat-capacity difference between forms. This difference is qualitatively consistent with the Brandts' model for protein unfolding. In the next paper[21] we present data over a wide pH and temperature range for transition I and evaluate the transition in terms of this model.

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Studies of the Chymotrypsinogen Family of Proteins. VII. Thermodynamic Analysis of Transition I of α-Chymotrypsin[1]

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Abstract: The reversible thermal unfolding of α-chymotrypsin has been studied in the acid pH region from 1.5 to 3.5 using ultraviolet difference spectroscopy. The thermodynamic characteristics of the transition are found to be qualitatively and quantitatively consistent with the Brandts model of protein unfolding since: (a) it has been found that the transition is a two-state transition characterized by a large, positive heat-capacity change; (b) there is found to be a temperature of maximum stability for the folded state; and (c) the single residue, thermodynamic parameters, $\Delta\bar{h}_h°$ and $\Delta\bar{s}_c°$, are the same for α-chymotrypsin as for chymotrypsinogen A. The differences in the thermodynamic changes for the thermal unfolding of these two proteins, and apparently for any proteins of this family, can be attributed primarily to differences in the size of their cooperative unfolding units. This conclusion is supported by other physical measurements. The implication of these results in relation to the thermodynamic determination of protein conformation and the use of thermodynamic studies of the protein conformation changes as a means for characterizing protein conformation is discussed.

In the preceding paper[2] we have shown that the conformational rearrangements of α-chymotrypsin (bovine) in the acid pH region can be analyzed in terms of two equilibria. The first of these, the transition from state A_a to state A_b, is characterized by only small changes in thermodynamic quantities, although it may be associated with the opening up of the buried ion pair between the carboxylate of aspartate-195 and the α-ammonium group of leucine-16.[3] This transition has thus

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