changes strongly suggests that the apparent value of $\Delta\bar{H}_A^\circ$ 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, $\Delta\bar{h}_h^\circ$ and $\Delta\bar{s}_c^\circ$, 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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(3) H. Parker and R. Lumry, to be submitted for publication.

far been detected only as a result of a specific pattern of change in the optical rotatory dispersion pattern attributable primarily to changes in a simple or complex Cotton effect centered near 207 nm.[4] It is also associated with changes in the 228-nm Cotton effect.[4-7] The second transition, transition I, which has large thermodynamic consequences, has been measured by spectral changes in the 290-nm region, by loss of high salt solubility, and by ORD changes. In view of the similarity of transition I for α-chymotrypsin to transition I for chymotrypsinogen A studied by Brandts[8,9] with a wide variety of observables, there is undoubtedly a number of additional changes in physical characteristics of the protein which could be used to follow transition I of α-chymotrypsin. Transition I has been shown to be a two-state transition at pH 2.0[10] and is thus amenable to thermodynamic analysis. In this paper we present data on transition I over a wide range of pH and temperature and analyze the transition in terms of the model of protein unfolding developed by Brandts.[9,11]

The Brandts model has been successfully applied to the thermally induced unfolding of chymotrypsinogen[9,11] and ribonuclease[12,13] and is summarized by eq 1 in

$$\Delta\bar{F}^\circ = p(N\Delta\bar{h}_h^\circ - NT\Delta\bar{s}_c^\circ + AT + BT^2 + CT^3) + \Delta\bar{F}^\circ_{elec} + \Delta\bar{F}^\circ_{titr} \quad (1)$$

which N is the total number of amino acid residues of the protein and p is an estimate of the fraction of the total residues which experiences change as a consequence of the unfolding process,[14] $\Delta\bar{h}_h^\circ$ and $\Delta\bar{s}_c^\circ$ are the temperature-independent enthalpy and entropy changes per residue; the polynomial $AT + BT^2 + CT^3$ measures the maximum contribution of the nonpolar side-chain interactions with water to the free energy changes as calculated from the differences in solubility of amino acids in water and 95% ethanol (the coefficients A, B, C which depend only upon the amino acid composition of the protein are given in the legend to Table II); $\Delta\bar{F}^\circ_{elec}$ is the change in $\Delta\bar{F}^\circ$ due to changes in the chloride ion concentration at constant pH and temperature; and $\Delta\bar{F}^\circ_{titr}$ is the change in $\Delta\bar{F}^\circ$ due to pH changes at constant chloride ion concentration and temperature. We will show that transition I of chymotrypsin conforms to the general picture of protein unfolding developed by Brandts and offer strong evidence that a phenomenological representation of this type is valid for many protein conformational transitions.

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(10) The most recent confirmation that transition I of α-chymotrypsin is a two-state transition has come from (a) I. Wadso, confirmation of the van't Hoff heat of the transition reported here by direct calorimetric measurements; and (b) the demonstration of the expected simplicity in the kinetics of the forward and backward rate processes corresponding to this transition by Pohl [F. M. Pohl, *European J. Biochem.*, **4**, 373 (1968)].
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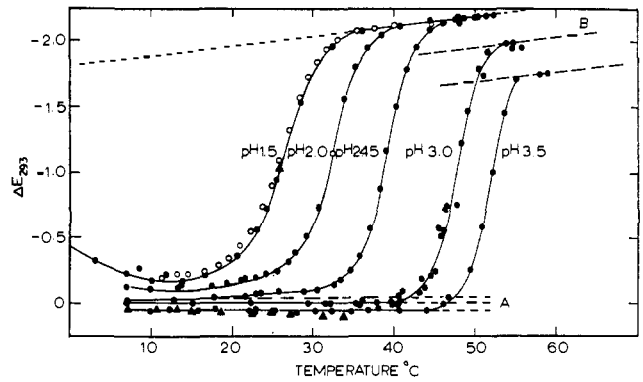

Figure 1. Temperature dependence of the difference extinction coefficient of α-chymotrypsin at several pH values. The various broken lines labeled A and B represent the values of $\Delta\epsilon_A$ and $\Delta\epsilon_B$ as a function of temperature for various pH values. Consult text for details.

Results

The difference extinction coefficient at 293 nm, $\Delta\epsilon$,[15] as a function of temperature is recorded for several pH values in Figure 1. The reference condition ($\Delta\epsilon = 0$) was selected as pH 3.0, 20°. In this figure the extrapolated variations of extinction coefficient with temperature for the pure low-temperature state, state A,[16] and pure high-temperature state, state B, are also given as dashed lines labeled A and B, respectively. It will be noticed that both $\Delta\epsilon_A$ and $\Delta\epsilon_B$ demonstrate a small monotonic change with pH when temperature is held constant. This change in absorption for the B state is probably an intrinsic property of the unfolded state rather than an indication that there are transitions among B substates as the pH is changed. Although the net-like structure of the "unfolded" polypeptide in the B state is undoubtedly sensitive to changes in pH and net charge, the observed changes in ϵ_B as pH is varied are most likely due to charge perturbations of the solvent-exposed chromophores as will be discussed later. We have been unable to detect substates of the B state in systematic studies of the ORD pattern as a function of pH and temperature, and, of course, if substates with significantly different thermodynamic properties existed, the several tests for two-state behavior would have failed. Although the tests do establish two-state behavior within error for α-chymotrypsin, the protein in state A exists as a mixture of two substates, A_a and A_b. However, the ultraviolet absorption spectra of the protein in the two substates are identical and the ORD measurements which reveal the transition $A_b \rightarrow A_a$ show that the enthalpy difference is only 4 kcal/mol. Had we been able to measure transition I starting with all the protein molecules in substate A_a and again starting with all protein in substate A_b, the enthalpy difference would be 4 kcal. Over the range of our experimental conditions, the expected maximum contribution to the enthalpy change of transition I due to change in these substate populations is less than 2 kcal, which is of the same size as the precision in the enthalpy determination. This contribution to $\Delta\bar{H}^\circ$ is undetec-

(15) The differential extinction coefficient is defined as the difference between the extinction coefficient at some set of conditions and the extinction coefficient at a reference set of conditions. For example, $\Delta\epsilon$ at 50°, pH 2.0 = ϵ(50°, pH 2.0) − ϵ(20°, pH 3.0).
(16) States A and B for α-chymotrypsin have been defined in the previous paper.[2]

table. Thus, while transition I can be experimentally treated as a two-state process, it should be noted that state A is actually an equilibrium mixture of substates A_a and A_b.

The magnitude and shape of the pH difference spectra at constant low temperature are very similar to the corresponding pH difference spectra obtained with lysozyme[17] and pepsin[18] but quite different from the difference spectra produced by a temperature change at constant pH. The latter show two minima, 293 and 285 nm, attributable at least in part to changes in the polarizability of the local environment of tryptophan and tyrosine chromophores, and the extinction-coefficient differences are large. Since the number of indole groups exposed during transition I can be measured independently, the maximum change in molar extinction coefficient at 293 nm can be shown to be about 1.0×10^3 mol^{-1} cm^{-1} per indole group. On the other hand, the pH difference spectra demonstrate a single minimum, at 291 nm, with a maximum difference in molar extinction coefficient per indole group of 2×10^2 mol^{-1} cm^{-1}. Such difference spectra obtained in studies of lysozyme and pepsin as a function of pH have been attributed to the perturbation of solvent-exposed indole and phenol groups by nearby charged groups, the perturbation being mediated through the solvent.[17] In the present work, the ratio of the change produced in $\Delta\epsilon_A$ by pH variation to that produced in $\Delta\epsilon_B$ by pH variation was found to be in approximate agreement with the ratio of "exposed" indole groups in the two states, A and B, when the latter ratio was determined by solvent perturbation using 20% sucrose–water solvents.[19] This comparison may be misleading since the indole groups exposed in state B probably respond in a different way to solvent variation than those exposed in state A. We also note that according to solvent-perturbation measurements the number of exposed tryptophan residues of chymotrypsin in state A is independent of pH from 2.0 to 7.0.[20] Thus the several results obtained in studies of the pH-produced difference spectra of α-chymotrypsin are consistent with the hypothesis that charge perturbation is the cause of the pH-induced absorption changes.

By measuring the temperature dependence of the 293-nm absorption of a pH 4.0 protein solution from 3 to 38° it was found that $\Delta\epsilon_A$ exhibits no temperature dependence. Below pH 2.5 α-chymotrypsin is at least partially in state B at all temperatures. This situation precludes direct measurement of $\Delta\epsilon_A$ at low pH values. It was thus necessary to assume that there is no pH dependence of $\Delta\epsilon_A$ below pH 2.5. This assumption is supported by the fact that $\Delta\epsilon_B$ is pH independent below 2.5 (see Figure 1). It is also strongly supported by high salt solubility measurements which will be discussed. $\Delta\epsilon_B$ exhibited a rather large temperature dependence which could only be determined directly for solutions at pH 2.5 or lower because of the onset of irreversible aggregation above 55°. Therefore, it was necessary to assume that the temperature dependence of $\Delta\epsilon_B$ was independent of pH above pH 2.5. This was found to be the case at pH 2.5 and below.

An important observation is a change in the sign of the temperature dependence of $\Delta\epsilon$ at about 12°. This is possible for a two-state process only if there exists an inversion temperature below which the standard enthalpy change for the transition is negative. This occurrence predicted by Brandts[11] is an important point in experimental verification of his model and will be discussed shortly.

All the transitions for which data are shown in Figure 1 were reversible. Below pH 3.0 the transition curves were reproducible without hysteresis to within 3%. At pH values of 3.0 and higher autolysis complicated the experimental situation.[19] Since autolysis produces spectral changes similar to those produced by transition I, certain precautions were necessary to ensure that our spectral data reflected the true equilibrium between states A and B. For this reason the data above pH 2.5 were obtained in the following way. A stock solution of the protein was kept at room temperatures under which conditions no autolysis was detectable by either irreversible ultraviolet-spectral changes or increases in the ninhydrin color. An aliquot of this solution was placed in the cuvette and quickly brought to the desired temperature and its difference optical density measured. In no case was the solution allowed to remain in the transition range for more than 1 hr.[21] Data were collected throughout the transition range so that experimental points for different aliquots overlapped on the temperature scale. In all cases the spectral change at 293 nm observed for different aliquots differed by no more than 0.003 optical density unit when measured at the same temperature.

Eisenberg and Schwert[22] have shown that chymotrypsinogen A in state B is insoluble in concentrated salt solutions and both they and Brandts have studied transition I using this observable as did Schellman for α-chymotrypsin.[23] However, the method has limited applicability because high protein concentrations are necessary and at some pH values aggregation and autolysis become serious complications. However, at pH 2.0, transition I is completely reversible[2] at all temperatures in the relatively concentrated solutions and at higher pH values if the temperature is kept below the transition temperature. It was thus possible to check our estimates of the pH dependence of $\Delta\epsilon_A$ and to establish that the apparent inversion temperature noted at low pH was a consequence of a true inversion in the transition. The fraction of protein in state B at several pH values was determined from precipitation measurements in 1.0 *M* KCl buffered at pH 3.0 at 4° according to the method of Eisenberg and Schwert.[22] The results are shown in Table I together with calculations of this fraction based on the ultraviolet spectral data. Good agreement is found from which we conclude that our estimates of the pH dependence of $\Delta\epsilon_A$ are reasonable. The inversion of transition I was detectable at pH 1.5 using solubility measurements but the change in solubility was only slightly larger than the error of measurement. The maximum high salt solubility was found to

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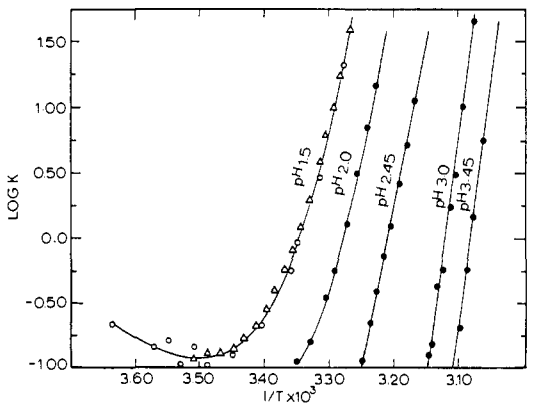

Figure 2. van't Hoff curves of the data in Figure 1. The open circles for pH 1.5 represent a duplicate experiment.

be at 10 ± 3° with the solubility decreasing as the temperature was lowered or raised from this point. In general, the fraction of the state B form determined from solubility measurements agreed with that calculated from the ultraviolet spectral changes within the aggregate errors of ±5%.

Table I. Fraction of α-Chymotrypsin in State B as a Function of pH as Determined by Loss of High Salt Solubility and Changes in Ultraviolet Absorption Spectrum

		Fraction in state B	
pH	Temp, °C	Solubility	Difference spectrum
1.5	14	0.13	
1.5	23	0.25	0.28
2.0	14	0.05	
2.0	23	0.09	0.10
2.5	14	0.00	
2.5	23	0.04	0.06
3.0	14	0.00	
3.0	23	0.00	0.00

The thermodynamic changes in transition I as a function of temperature and pH can be calculated from eq 1–4 given in the previous paper[2] using the appropriate values of $\Delta\epsilon_A$ and $\Delta\epsilon_B$ taken from Figure 1. These calculations yield the van't Hoff plot shown in Figure 2. At the lower pH values distinct curvature is noted, and the inversion temperature is clearly seen at approximately 12°. The temperature dependence of the apparent enthalpy change for the transition at all pH values is seen to be such that $\Delta\bar{H}°$ monotonically increases with temperature. This observation is consistent with the two-state approximation[24] which has been established for these calculations and which was established for transition I at pH 2.0.[2]

To obtain precise values for the fitting parameters of eq 1 by least-squares procedures the data must have high accuracy and must cover the widest possible temperature range. The data obtained at single pH values are not in general sufficient to satisfy these requirements. Brandts[9] observed with transition I of chymotrypsinogen that the apparent standard enthalpy change for the transition was independent of pH and ionic strength within small errors so that data obtained at

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

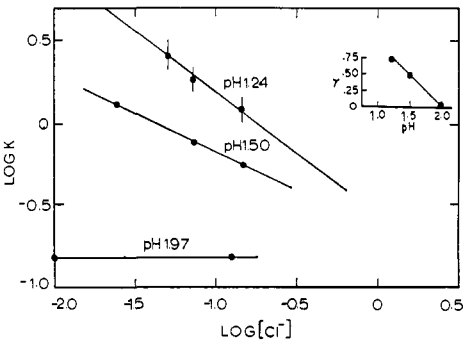

Figure 3. Log K *vs.* log [Cl^-] for transition I of α-chymotrypsin at selected, low pH values. Inset shows behavior of γ as function of pH. See text for details.

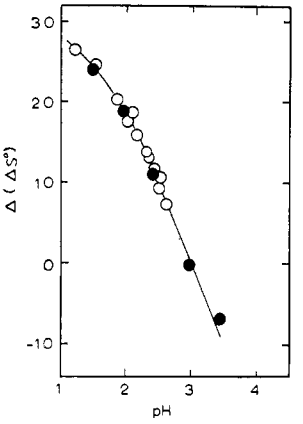

Figure 4. Variation of $\Delta\bar{F}°$ as function of pH at constant temperature and chloride ion equal to 0.01 *M*. Solid line corresponds to fit with eq 3.

different pH values could be easily adjusted to a single set of solvent conditions. The results (Figure 2) show this also to be the case for α-chymotrypsin. Comparison of the $\Delta\bar{H}°$ values obtained from the slopes of van't Hoff plots for transition I at different pH values (Figure 2), but at identical temperatures, show that $\Delta\bar{H}°$ is independent of pH within our errors. Thus, all experimental data can be adjusted to any arbitrary set of solvent conditions by determination of the pH and chloride ion dependencies of the standard free energy change of transition I. These can be determined from the data in Figure 2 taken together with the measurements of the pH and chloride ion dependencies of the extinction-coefficient difference at constant temperature (Figures 3 and 4).

Figure 3 summarizes the data showing the effect of chloride ion on the equilibrium of transition I. A linear relationship between $\Delta\bar{F}°$ and log (Cl^-) was observed. For chymotrypsinogen unfolding Brandts[9] observed that the phenomenological effect of chloride ion concentration on the equilibrium could be represented by

$$\frac{\Delta\bar{F}°_{elec}}{RT} = -\gamma \log \frac{(Cl^-)}{0.01} \qquad (2)$$

where γ is determined experimentally and is a complex function of pH. In the present case, however, we do not wish to interpret this effect, but only to use the form of eq 2 to correct all data to a single set of solvent con-

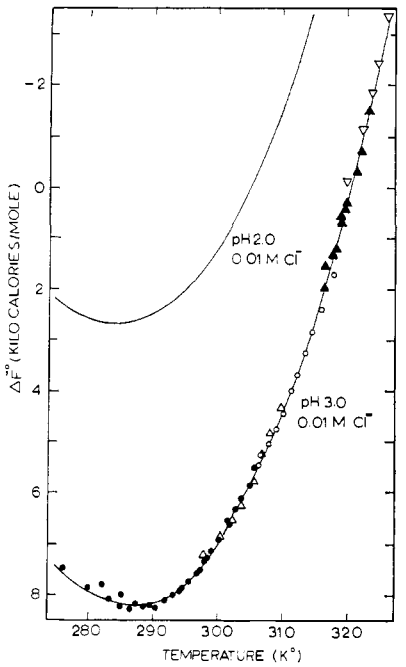

Figure 5. Variation of $\Delta\bar{F}^\circ$ with temperature at pH 2.0 and 3.0. The various symbols (*e.g.*, △) represent results obtained at different pH values. Solid line corresponds to fit of eq 1 to data with parameters given in Table II. The deviations of the experimental points from the least-squares line are well within the errors of these points calculated from the estimated errors in ϵ, ϵ_A, and ϵ_B.

ditions. All data in Figure 3 were obtained in 0.01 *M* chloride ion except at pH 1.5 where the chloride ion concentration was necessarily higher. Therefore, we need only correct the free energy profile at pH 1.5 for the effect of extra chloride ion. In this case it was found that $\gamma = 0.50$.

It is not generally possible to determine from van't Hoff studies the correct form of the pH dependence of $\Delta\bar{F}^\circ$. Brandts[9] found he could represent such data for chymotrypsinogen by eq 3 in which n is the apparent number of acid groups linked to the transition

$$\Delta\bar{F}^\circ_{titr} = -nRT \ln [1 + (a_H/K_B)]/[1 + (a_H/K_A)] \quad (3)$$

with K_A and K_B the acid ionization equilibrium constants for these n acid groups in state A and state B, respectively, and a_H is the hydrogen ion activity. Equation 3 is a condensed form of Brandts' general equation which is adequate for present purposes and should be entirely satisfactory if the effective K_A and K_B values of all n groups are identical. It was assumed that K_B was equal to 3×10^{-5} *M*, the ionization constant for a normal carboxyl group. K_A and n could then be estimated and it was found that the form of eq 3 provided a good representation of the pH data for α-chymotrypsin. The pH-dependent part of $\Delta\bar{F}^\circ$, $\Delta\bar{F}^\circ_{titr}$, measured at all conditions provided a set of differential free energies, $\Delta(\Delta F^\circ_{titr})$, which could be used to adjust $\Delta\bar{F}^\circ$ data to any desired pH value within the experimental pH range. The variation of $\Delta\bar{F}^\circ$ with pH is shown in Figure 4 with the arbitrary choice that $\Delta(\Delta\bar{F}^\circ_{titr}) = 0$ at pH 3.0. Since $\Delta H_{titr} \sim 0$, the data have been represented in terms of $\Delta(\Delta S^\circ)$ with $\Delta(\Delta S^\circ) = 0$ at pH 3.0. The data obtained below pH 2.0 have been corrected for chloride ion dependence using eq 2. The value of γ at the various pH values has been either directly obtained from the data in Figure 3 or by linear interpolation of the pH dependence of γ to intermediate values of pH. The filled circles in Figure 4 are points calculated from the data in Figure 2. The solid line represents the least-squares fit of the data to eq 3 with parameter values of $n = 5.7$ and $K_A = 3 \times 10^{-2}$ *M*. The fit of the two-parameter equation is within the estimated error of the experimental points.

The pH and ionic strength dependence of $\Delta\bar{F}^\circ$ can be phenomenologically adjusted in the manner described so that all data apply to changes in $\Delta\bar{F}^\circ$ under the same single set of solvent conditions. The standard free energy change as a function of temperature shown in Figure 5 was obtained by adjustment of all data to pH 3.0 and 0.01 *M* chloride ion using the method just outlined. All adjustments were entropy adjustments since the total enthalpy change, $\Delta\bar{H}^\circ$, was found to be independent of pH and chloride concentration; the only effect of these solvent changes is to shift the position of the transition on the temperature scale.

The solid line in Figure 5 is the least-squares line using a linear version of eq 1 to fit the data (see eq 4)

$$\Delta\bar{F}^\circ = \alpha + \beta T + \gamma f(T) \quad (4)$$

where, in terms of eq 1, $\alpha = pN\Delta\bar{h}^\circ_h$, $\beta = -pN\Delta\bar{s}^\circ_c + pA + (\Delta\bar{F}^\circ_{elec}/T) + (\Delta\bar{F}^\circ_{titr}/T)$, $\gamma = pB$, and $f(T) = T^2 + (C/B)T^3$). [Note that $\Delta\bar{F}^\circ_{elec}/T$ is equal to zero at our ionic strength (0.01 *M* Cl^-) and that $\Delta\bar{F}^\circ_{titr}/T$ is independent of temperature. Both these terms are independently evaluated prior to fitting the data to eq 4.] The parameters obtaining from this fitting procedure are recorded in Table II along with previous values obtained for chymotrypsinogen. The deviation of the best-fit line is less than experimental error at all points (see caption for Figure 5). It should be noted that the free energy changes are less accurate for α-chymotrypsin than for chymotrypsinogen. As previously noted, these larger errors result from the fact that at lower pH values solutions of chymotrypsin always contain some molecules in state B regardless of temperature. This situation necessitated the use of a biased weighting function for the experimental data. The error of each $\Delta\bar{F}^\circ$ was estimated in the usual manner with the following error estimates for the difference extinction coefficients: at pH 2.5 and above these error estimates, $\delta(\Delta\epsilon)$, $\delta(\Delta\epsilon_A)$, and $\delta(\Delta\epsilon_B)$, were 0.05, 0.05, and 0.10, respectively; below pH 2.5 $\delta(\Delta\epsilon)$, $\delta(\Delta_A)$, and $\delta(\Delta\epsilon_B)$ were estimated to be 0.05, 0.10, and 0.10, respectively; the error in the temperature (±0.02°) can be neglected.

In addition to the above errors other errors, which were primarily nonrandom, were introduced by correction of all data to identical solvent conditions.

Table II. Thermodynamic Parameters for Transition I of α-Chymotrypsin and Chymotrypsinogen at pH 3.0[a]

	p	$\Delta\bar{h}^\circ_h$, cal/mol	$\Delta\bar{s}^\circ_c$, eu
α-Chymotrypsin	1.01 ± 0.02[a]	780 ± 10	5.1 ± 0.2
Chymotrypsinogen A (5)	0.63	800	5.1

[a] These parameters have been calculated with eq 1 using values of −2270.2, 18.122, and −0.02792 for *A*, *B*, and *C*, respectively.

These systematic errors could not be directly detected or estimated, but their importance was tested by least-squares fitting of various combinations of sets of experimental data corrected to pH 3.0 and 0.01 *M* chloride ion (*e.g.*, the data obtained at pH 2.5, 3.0, and 3.5 were fit as a group). In all those cases in which at least two sets containing at least 20 experimental points were used, the adjustable coefficients agreed within 5% with those obtained using all the data. Examples of results with various combinations of experimental data are shown in Table III. These results show that our

Table III. Thermodynamic Parameters Using Various Sets of Data

Data, pH	p	$\Delta\bar{h}_h°$, kcal/mol	$\Delta\bar{s}_c°$, eu
1.5, 2.0, 2.5	1.02	770	5.1
2.5, 3.0, 3.5	1.02	770	5.0
2.0, 2.5, 3.0	1.04	760	5.0

biased weighing function did not distort the statistical significance of the data and that our adjustment procedure to identical solvent conditions was accurate within experimental error. In addition, these results show that the temperature dependence of the thermodynamic quantities observed at higher pH and hence higher temperatures are consistent with those observed at the lower pH and hence lower temperatures. For example, the parameters obtained using only the data at pH 2.5, 3.0, and 3.5 correctly predict the temperature of maximum stability and the apparent enthalpy change for transition I as observed at pH 1.5.

Discussion

Definition of State A. State A has been explicitly defined as a mixture of states or substates A_a and A_b[2] plus any other intermediate substates which may exist. Since state A is a mixture of thermodynamic states, changes in the distribution between A_a and A_b may be reflected in changes in physical observables as is indeed the case in ORD measurements. However, the observables used to measure transition I, loss of solubility in high salt buffer and ultraviolet absorption spectrum, do not distinguish between states A_a and A_b. It can be readily shown that tests for two-state behavior using variables which cannot distinguish the differences between substates will give positive evidence for two-state behavior. For example, there will be no maximum in the apparent enthalpy of transition I.[24] The heat-capacity change in transition I will be anomalous since it will contain "between-states" contributions of the substates as well as the true "within-states" heat capacities. That is, the enthalpy associated with the variation of relative proportions of A_a and A_b substates will appear as an anomalous contribution to the heat-capacity change of transition I. Fortunately, in the present case the "between-states" heat-capacity contribution is small since the standard enthalpy difference between substates A_a and A_a is only 4 kcal/mol.[2] Within the precision of this work and the currently highest precision possible in calorimetric determination of $\Delta\bar{H}°$ and $\Delta\bar{C}_p°$[10,25] transition I of α-chymotrypsin has the phenomenological characteristics of a two-state transition at pH 2.0 and we have given evidence that this feature is preserved throughout the acid pH region above pH 1.5.

Validity of the Brandts Representation. The form of eq 1 is such that the total free energy of unfolding, apart from $\Delta\bar{F}°_{elec}$ and $\Delta\bar{F}°_{titr}$, is estimated in terms of contributions from individual amino acid residues. This is a practical requirement which should not be confused with the real situation. The most simple secondary interactions such as hydrogen bonds may be adequately considered as involving only two residues. The more complicated interactions involve clusters of residues and peptide links. Such is the case with van der Waals' dispersion interactions within proteins which determine the effective force constants for low-frequency vibrations spread over many atoms of the protein. Although it is impossible to separate all the interactions of importance in protein folding in a manner such that their individual contributions to the free energy can be determined, one can classify the interactions empirically using the individual residues as a bookkeeping device in the manner of Tanford[26] and Brandts.[11] Even in this case it is not possible to speak of specific residues, but it is necessary to define one or more different classes of average residue. In this way, the averaging process over all different types of residues includes both the total number of residues, and also differences in the populations of the various classes of residues. The disadvantage of this procedure is that the final average residue determined *via* Brandts' procedure which uses the total amino acid composition of the protein may not be correct in partial unfolding processes. In other words, the average residue of the protein as a whole may not be the average residue of the cooperative unfolding unit.[14] This will be the case whenever the cooperative unit is small. If it were possible to establish an exact description of the cooperative unfolding regions and the surfaces of confinement of these regions by the remainder of the molecule, more appropriate averages could be calculated for a given conformational transition. At present, this information is not available and one must assume that the whole protein average is an appropriate average for the cooperative unit under consideration. These aspects of the problem will be considered in a more quantitative fashion at a later point.

Detailed interpretation of the physical meaning of $\Delta\bar{F}°_{elec}$ and $\Delta\bar{F}°_{titr}$ as given in eq 1 cannot yet be made,[14] but these contributions to $\Delta\bar{F}°$ can be evaluated experimentally by determination of the change in free energy as a function of the ionic strength at constant temperature and pH and the change in free energy as a function of pH at constant ionic strength and temperature. Since $\Delta\bar{F}°_{elec}$ and $\Delta\bar{F}°_{titr}$ for both chymotrypsinogen[9] and chymotrypsin are entropic, only an adjustment in the linear term in the temperature polynominal (eq 4) was required for conversion of all data to a constant ionic strength and pH. Furthermore, since $\Delta\bar{F}°_{elec}$ and $\Delta\bar{F}°_{titr}$ under defined conditions can be estimated empirically, the only parameters which need be evaluated by least-squares fitting of the free energy data are p, $\Delta\bar{h}°_h$, and $\Delta\bar{s}°_c$.

(25) L. Benjamin and A. Truman Schwartz, personal communication, 1967.

(26) C. Tanford, *J. Am. Chem. Soc.*, 86, 2050 (1964).

Before consideration of the general implications of the Brandts representation, let us look into the details of the experimental support for this model. First, the form of eq 1 implicitly establishes the form of the heat-capacity change on unfolding

$$\Delta\bar{C}^\circ_p = -p(2BT + 6CT^2) \quad (5)$$

Since B and C are obtained from the transfer free energies of the nonpolar residues of the protein,[11] it follows that the heat-capacity change is due entirely to changes in the exposure of nonpolar groups to the aqueous environment upon unfolding. Heretofore, this change in heat capacity has only been observed in a strong temperature dependence of the van't Hoff heat for unfolding calculated assuming two-state behavior. The validity of this latter assumption has been verified for several members of the chymotrypsinogen family of proteins as well as ribonuclease.[24] Recently the heat capacity of the unfolded and folded forms of chymotrypsin has been measured.[25] The difference in heat capacity of states A and B measured calorimetrically agrees within experimental errors with the heat-capacity change determined by the van't Hoff method in this work, although the errors are approximately ±2 kcal/(mol deg). More recently $\Delta\bar{H}^\circ$ for transition I at 40° has been measured calorimetrically[10] and was found to be 116 ± 3 kcal/mol which may be compared to 120 kcal/mol found in this study. These agreements support the two-state assumption and also verify Brandts' prediction (from eq 1) that the heat capacity of a protein increases on unfolding.

The form of eq 1 also implies that at some temperture, T_{max}, the free energy change for unfolding will be maximum, and that this temperature, *i.e.*, that at which $(d\Delta\bar{F}^\circ/dT)_p = -\Delta\bar{S}^\circ = 0$, is primarily a function of the average amino acid residue and not of the size of the cooperative unfolding unit as determined by the magnitude of p, the relative size of the cooperative unit.[27] Brandts had originally calculated from his high-temperature data for the thermal unfolding of chymotrypsinogen that the temperature of maximum stability, T_{max}, should be observed at approximately 12°.[11] Because of experimental limitations with that system he was unable to verify this prediction in strictly aqueous solution.[9,11] We have found experimentally that the temperature of maximum stability of α-chymotrypsin and also dimethionine sulfoxide–α-chymotrypsin[19,28] is approximately 12° as is required by the Brandts model since the amino acid compositions of these three proteins are essentially identical. This finding is of considerable importance since it provides experimental validation of the average residue approximation in Brandts' analysis.

Equation 1 is a special case of Brandts' analysis in which it is assumed that the cooperative unit for the unfolding transition has the same average residue composition as the total protein. If this assumption is approximately correct, and it certainly is never exactly correct unless the cooperative unit is the total protein, the thermodynamic changes in the transition can be estimated as equal to the total change for unfolding of the entire protein times the fraction, p, of total residues in the cooperative unit. To test the validity of this assumption using a single protein it is necessary to have independent estimates of the thermodynamic changes for the individual residues of the protein and an independent estimate of p for the protein chosen. This is, at present, impossible. However, by comparing different proteins of the same parent family the degree of validity of the average residue approximation can be established without any requirement that the specific values of the thermodynamic changes estimated in the procedure have any absolute reliability. This procedure is based on the assumption (insofar as the assumption is correct) that $\Delta\bar{h}^\circ_h$ and $\Delta\bar{s}^\circ_c$ will be essentially constant[29] for this protein family so that all variations in transition I behavior can be attributed to differences in p and only in p. A comparison of this type for chymotrypsin and chymotrypsinogen is shown in Table I and demonstrates that the average residue approximation is quantitatively satisfactory at the present level of precision. Several additional members of the chymotrypsinogen family have been investigated recently and give similar good agreement.[6,30] The values obtained for $\Delta\bar{h}^\circ_h$ and $\Delta\bar{s}^\circ_c$ depend on the choice of the temperature function (*e.g.*, $T^2 + (B/C)T^3$), incorporated into Brandts' formalism to take account of the interactions between nonpolar residues and water. The polynominal in T used in constructing eq 1 will be replaced by a more refined temperature function when better models for solvation of hydrophobic side chains become available. Different p values and different values of $\Delta\bar{h}^\circ_h$ and $\Delta\bar{s}^\circ_c$ will result from applications of different functions of this sort. However, so long as the heat capacity change in transition I is included as due to hydrophobic solvation effects and no others, the variation among the parameters within a single protein family will appear predominantly in p.

The absolute value of p for a given protein will also vary with the specific choice of temperature dependence in expressions for $\Delta\bar{F}^\circ$ (*cf.* eq 4). Using eq 1 and the A, B, and C values computed from transfer expressions for the individual amino acid side chains of chymotrypsinogen, p is about 1 for α-chymotrypsin and about 0.6 for chymotrypsinogen. Although the absolute values of these quantities are unlikely to be correct[14] (*vide infra*), the relative magnitudes are apparently correct. This conclusion plus our inability to detect differences between the states B of α-chymotrypsin and chymotrypsinogen by a variety of experimental methods[19] suggest that the conformations of these two proteins must be different in state A. If so, chymotrypsinogen is less completely folded by the heat capacity criterion implicit in the use of the p parameter as a measure of the size of cooperative units. This conclusion is sup-

(27) The actual position of the temperature of maximum stability for a given protein family also depends upon $\Delta\bar{F}^\circ_{titr}$ and $\Delta\bar{F}^\circ_{elec}$ which may vary from protein to protein within a given family, but the change in T_{max} produced by this variation is at most only a few degrees. Furthermore, T_{max} may vary significantly from protein family to protein family since it is strongly dependent upon the average hydrophobic residue. For instance, T_{max} for the chymotrypsinogen family has been found to be in the vicinity of 10–13° for all species, but the T_{max} calculated for ribonuclease lies below 0° and is thus not experimentally observable under normal conditions.

(28) R. Biltonen and R. Lumry, to be submitted for publication.

(29) This assumption is not unreasonable at this level of approximation insofar as the interactions contributing to these terms are dominated by non-side-chain interactions. For example, the conformational entropy is primarily, though certainly not totally, dependent upon the freedom of rotation about the bonds of the "unfolded" backbone. At higher levels of approximation, the assumption is obviously not valid and the value of these contributions will have to be estimated residue by residue.

(30) R. Biltonen and R. Lumry, *J. Am. Chem. Soc.*, 87, 4209 (1965).

ported by deuterium-exchange studies in which it has been found that chymotrypsinogen has a smaller hard to exchange core of protons than α-chymotrypsin.[19,31] It appears to be confirmed by recent nmr studies in which it has been found that chymotrypsinogen in state A has a larger amount of segmental flexibility than α-chymotrypsin.[32,33]

In another place[14] we have considered what literal interpretation can be placed on the parameter p and find that p is a good relative estimate of the fraction of nonpolar residues which undergo changes of state in transition I. Thus, the p value of about unity obtained for α-chymotrypsin using Brandts' original estimates for side-chain transfer free energies indicates that about half of the total residues and some fraction greater than 0.5 of the nonpolar residues participate in transition I of this protein. We have also examined the heat capacity expression derivable from eq 1 and it is found that this expression can be written as eq 5 in which C/B is a constant independent of the protein so that the function $(T + 3(C/B)T^2)$ is a universal function of water which measures the response of bulk water to dissolved nonpolar groups or molecules. These conclusions are based on the validity of eq 1. Recently Shiao[6] and Fahey[34] have provided evidence that eq 1 is overelaborate and that the heat capacity change in transition I can be adequately described as a constant until such time as van't Hoff and calorimetric precision becomes considerably better. However, the conclusion that the heat capacity change is due entirely to changes in solvation of nonpolar side chains of proteins is in no way compromised by the new simplification. Hence, the experimental heat capacity change can be treated as a constant single-residue quantity characteristic of the average residue multiplied by the number of average residues in the cooperative unit. An alternative way to present the data is in terms of a reference hydrocarbon. If we choose propane as this reference substance the hydrophobicity of the cooperative unit of transition I for α-chymotrypsin is equivalent to 50 ± 20 propane molecules.[14,35] The standard heat capacity change is 5 ± 2 kcal/(mol deg) at 25° according to the best calorimetric information available[25] so that at this temperature the contribution per propane unit is about 0.10 kcal/(mol deg). The heat capacity of the total protein in the dry solid and in solution is 10 ± 1 kcal/(mol deg) also determined calorimetrically.[10,25] On a specific heat capacity basis the hydrophobic contribution to the heat capacity of α-chymotrypsin in state B is 0.2 cal/(g deg) so that the total heat capacity at 25° is 0.6 cal/(g deg) in state B and 0.4 cal/(g deg) in state A. These numbers undoubtedly have considerable error despite the fact that they were obtained with the best experimental precision now available.

Our results individually and in aggregate provide strong support for the method of analysis of cooperative unfolding data for protein systems suggested by Brandts and based on the special role of the heat capacity change in such processes as a measure of the change in nonpolar groups exposed to solvent At the present stage of its development, the Brandts analysis divides the total free energy change in an unfolding transition into five phenomenological classes: the pH-dependent contribution, the salt-dependent contribution, the hydrophobic contribution, the temperature-independent enthalpy change $p\Delta\bar{h}^\circ_h N$, and the temperature-independent entropy contribution $pN\Delta\bar{s}^\circ_c$. These classes cannot all be independent if they are judged in terms of their makeup of conventional bonding concepts such as hydrogen bonding or van der Waals' interactions. These latter bonding concepts are not, however, pragmatically sound in a problem of the complexity of protein conformations, and it is considerably less misleading to use the classes of Brandts in comparing the conformational thermodynamic situation in one protein with that in another protein. These classes of practically obtainable data rest on a reasonably straightforward empirical foundation. As new independent variables appear in protein study, additional phenomenological classes can be recognized. At present, the fitting parameter $\Delta\bar{h}^\circ_h$ contains contributions from hydrogen-bonding changes, changes in van der Waals' interactions, hindered rotation, longer range dipole–dipole interaction, and so on. As an average quantity it should be very nearly the same for a variety of soluble proteins. The single-residue average quantity $\Delta\bar{s}_c$ contains all the temperature-independent entropy contributions, but is heavily dominated by the conformational entropy change. This quantity too should be very similar among the water-soluble proteins. Variations in all these quantities provide the practical means for comparing one protein with another and for pinpointing the sources of abnormal thermodynamic changes, as well as the effects of change in character of the solvents, *i.e.*, use of urea–water, glycerol–water, alcohol–water solutions, etc., in terms of the parameters which change.[9,36]

The unique relationship between heat capacity change and change in exposure of hydrophobic groups during a cooperative transition of a protein provides a pragmatically sound way to distinguish among broad classes of such processes. Thus, an unfolding process can be distinguished by an increase in the heat capacity. In fact, it should be possible in the near future to estimate quite accurately the extent of such an unfolding process in the manner described here. Processes which involve only expansion or contraction should have no significant heat capacity change nor should they have unusually large activation enthalpies and entropies. Such processes, called "subtle changes," have now been found and are discussed elsewhere.[14] Processes which involve unfolding followed by refolding may not show significant changes in heat capacity, but they are likely to have large activation enthalpies and entropies. They may also in general prove to be slow.[14]

Future developments along the lines pioneered by Brandts are likely to deemphasize the use of model transfer reactions in order to concentrate on the heat capacity changes observed in protein reactions themselves. In other words, it is probable that for some time to come proteins will be less misleading models for other proteins than the small molecule models. These

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(36) J. F. Brandts in "Biological Macromolecules," S. Timasheff and G. Fasman, Ed., Marcel Dekker, Inc., New York, N. Y., 1969.

matters have been discussed in another place.[14] In addition, it now appears that the heat capacity expression of Brandts derived from eq 1 requires revision.

Experimental Section

The experimental details of this work have been described.[2]

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Conformations of Cyclic Peptides. III. Cyclopentaglycyltyrosyl and Related Compounds[1]

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Abstract: Proton magnetic resonance studies of cyclopentaglycyl-L-tyrosyl and a partially C-deuterated derivative, using water, dimethyl sulfoxide, and trifluoroacetic acid as solvents, show that the six amide protons of this peptide are divided into two groups. Two protons are shielded from the solvent and four are exposed to it. This observation strongly supports an internally hydrogen-bonded structure, shown in Figure 1, as the stable form of the cyclic hexapeptide backbone. To this stable backbone conformation the single side chain is attached at one of the "corner" positions (1, 3, 4, and 6 in Figure 1), at least in dimethyl sulfoxide or a dimethyl sulfoxide–water mixture. Additional evidence is adduced from the spectra to define more narrowly, although with less certainty, details of the location and conformation of the side chain. The syntheses of the cyclic peptides are described.

Continuing our application of proton magnetic resonance to problems of peptide conformation in solution, we have taken up the study of cyclic hexapeptides, which are the most readily accessible cyclic peptides after diketopiperazines, and, in distinction to the latter, may pose significant questions of peptide backbone conformation. In this report we describe the syntheses and discuss conformational inferences from the proton spectra of cyclopentaglycyl-L-tyrosyl (I) and a partially deuterated derivative, *c*-Gly-Gly-Gly-d_2-Gly-d_2-L-Tyr (II). For these peptides there are two main questions of conformation: the shape of the peptide backbone and the arrangement of the side chain. We have reached definite conclusions about the first, and more tentative ones about the second.

The secondary structure of a cyclic hexapeptide ring undoubtedly depends on its substituents, but the single form that has so far received the most support was originally put forth by Schwyzer.[2] This structure, originally suggested on the basis of the facile cyclodimerization of carboxyl-activated tripeptide derivatives, is illustrated schematically in Figure 1. In it, all of the amide groups are planar and *trans*, and there are two transannular hydrogen bonds. A structure like this does in fact appear in an X-ray crystallographic analysis of cyclohexaglycyl hemihydrate.[3] Although the crystal examined contains four different conformations of the peptide ring, all with planar, *trans* amide groups, and all involved in intermolecular hydrogen bonds, one conformation, the only one containing intramolecular hydrogen bonds, does have the two hydrogen bridges of the Schwyzer structure.

Something close to the Schwyzer structure also appears in the crystal of ferrichrome A, a naturally occurring cyclic hexapeptide that has three hydroxamic acid side-chain groups coordinated to a ferric ion.[4] Here half of the cyclic hexapeptide ring has the Schwyzer structure, *i.e.*, there is one ring of nine heavy atoms closed by a transannular amide–amide hydrogen bond, but the other half is somewhat distorted by the requirements of a hydrogen bond between the peptide backbone and the side-chain hydroxamate complex. Distortion of the backbone by side-chain interactions is likely to be fairly common in heavily substituted cases. In fact, infrared studies of a group of diastereoisomers of *c*-Gly-Phe-Leu-Gly-Phe-Leu have been interpreted to indicate that, if sufficiently bulky substituents are present on the same side of the hexapeptide ring, some of the peptide bonds are constrained to the *cis* configuration.[5] This suggestion of steric interference between side chains is consistent with cyclization yields, which decrease with increasing numbers of *cis* side chains.[6] The molecule reported on in this paper, having only one side chain, should retain the conformational backbone preference of the unsubstituted cyclohexaglycyl backbone.

A priori, if a particular transannularly hydrogen-bonded structure is strongly favored for the cyclic hexapeptide backbone in solution, it should be revealed by studies of the exchange rates and/or chemical shifts of the amide protons. The bridging amide protons, being

(1) (a) This work was supported by research grants from the National Science Foundation, GB 4514, and the National Institute of General Medical Sciences, U. S. Public Health Service, GM 14069. (b) Paper II of this series is K. D. Kopple and M. Ohnishi, *J. Am. Chem. Soc.*, **91**, 692 (1969).

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