Thermodynamics of Protein Denaturation. A Calorimetric Study of the Reversible Denaturation of Chymotrypsinogen and Conclusions Regarding the Accuracy of the Two-State Approximation *

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ABSTRACT: The calorimetric properties of chymotrypsinogen in dilute solution have been investigated in the pH range from 2 to 3 in a sensitive differential heat capacity calorimeter. The heat capacity of the native protein is found to be 0.40 cal g^{-1} deg^{-1} at 20°, and it exhibits a strong positive temperature coefficient from 0 to 25°. Heat capacity measurements through the temperature region where the reversible denaturation occurs permit estimates of $\Delta H°$ and ΔC_p for the process of unfolding. Calorimetric values of $\Delta H°$ vary from about 100 kcal $mole^{-1}$ at 42° (pH 2) to 140 kcal $mole^{-1}$ at 54° (pH 3) while ΔC_p values change from about 3800 to 2800 cal $mole^{-1}$ deg^{-1} over the same range of conditions. The comparison of these calorimetric values with estimates made from the two-state analysis of transition curves shows no significant discrepancies in either the $\Delta H°$ or the ΔC_p parameters. It is concluded from this that the chymotrypsinogen denaturation obeys the two-state approximation to a high degree of accuracy under the conditions used in this study.

The use of the two-state approximation to describe protein denaturation reactions dates back at least 35 years (Anson and Mirsky, 1934). The existence of a controversy concerning the validity of this simple mechanism is clearly evident in the early review of protein denaturation by Neurath *et al.* (1944) who argue against an "all-or-none" unfolding mechanism, *i.e.*, a two-state mechanism which restricts the two states to the completely native and the completely unfolded polypeptide chain. More recently, a less restrictive picture of the two-state mechanism (Lumry *et al.*, 1966; Hill, 1963; Brandts, 1969) has evolved in which it is recognized that the two states need not be the extreme states of folding and that they may, in fact, be dynamic states whose average properties will change with changes in temperature, pressure, or solvent composition due to thermodynamic redistribution. Although this more liberal view of the two-state mechanism has attracted substantial support, particularly for the denaturation reactions of certain small proteins, there still exists some question about its quantitative validity (Poland and Scheraga, 1967; Gill and Glogovsky, 1965; Klee, 1967) and it seems likely that this uncertainty will continue for some years to come. Simple theoretical treatments of protein unfolding reactions are of no help in resolving the problem since they also lead to conflicting conclusions (Poland and Scheraga, 1967; Brandts, 1969) regarding the reasonableness of the two-state mechanism, depending upon what assumptions are incorporated into the treatments. It seems likely that the applicability or lack of applicability of the two-state mechanism will have to be established from experimental information on individual denaturation reactions, and that some proteins will be found to adhere more closely to this simple mechanism than will others.

The reversible denaturation of chymotrypsinogen has been studied previously in our laboratory (Brandts, 1964a,b) and the analysis of the data was based on the two-state approximation. The studies reported in this paper were designed to provide more definitive information about the validity of the approximation for chymotrypsinogen, making use of calorimetric measurements. The comparison of thermodynamic parameters obtained from direct calorimetric measurements with the equivalent parameters determined indirectly by the two-state analysis of transition curves is capable of providing a critical test of the quantitative reliability of the approximation (Lumry *et al.*, 1966; Brandts, 1969; Tanford, 1968).

Experimental Section

Protein solutions were prepared from commercial chymotrypsinogen (Worthington Corp., batch 8HA). Each solution was extensively dialyzed against the appropriate solvent prior to making calorimetric measurements. For solutions below pH 2.5, the solvent contained only HCl and no buffer or supporting electrolyte. At pH 2.5 and above, the solvent was 0.05 M glycine-HCl. Protein concentrations where determined spectrophotometrically, using an extinction coefficient of 19.7 at 282 nm for a 1% (w/w) solution in a 1-cm cell. This extinction coefficient was the average of ten determinations by dry weight analysis. For calculation of the calorimetric parameters, a molecular weight of 25,700 was used (Tanford, 1968).

The calorimeter used in this study, to be described in detail at a later time, was built in our laboratory and is the differential heat-capacity type. Twin platinum cells of 5-ml capacity

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are filled with nearly equal weights of protein solution and solvent. Each cell is in thermal contact with a main and an auxiliary heater. The carefully matched main heaters for the two cells are connected in series to a regulated DC power supply, providing nearly identical power to the solvent and solution continuously. Any temperature difference which may develop between the cells is detected by two 10-junction thermopiles. This error signal drives a feedback circuit which supplies current to the auxiliary heater of the thermally lagging cell, keeping the temperature difference between the two cells to within 25 × 10^{-6}° of null. The auxiliary heater current is squared electronically and recorded continuously on a strip chart recorder.

The twin cells are suspended in a carriage which fits snugly in an aluminum adiabatic shield maintained to within 125 μdeg of the average cell temperature by means of a thermopile-driven feedback circuit and heaters. The adiabatic shield is mounted in a water-tight vessel which is submerged in a water bath maintained by use of a differential controller to within 0.005 deg of the calorimeter cells.

A heating rate of 10 deg/hr was used in these experiments. The temperature of the calorimeter system was measured with a 25-junction thermopile and recorded continuously on second strip chart recorder, so that the instrument provides continuous values of the differential power as a function of temperature.

For each protein experiment a base line was obtained with equal weights of the appropriate solvent in the sample and reference cells. These base lines, which showed a small and completely reproducible temperature dependence, were subtracted from the protein data prior to the calculation of heat capacities.

The constant for converting recorder output into differential heat capacity was calculated from the various resistances and voltages. The value of this constant was checked by both an electrical calibration and also by measuring the differential heat capacities of water samples of various weights. These three methods of standardization were in agreement to better than 2%.

Results

Most of the calorimetric studies of protein denaturation in the past have been carried out at protein concentrations from 1 to 10%. Although these high concentrations usually provide fairly good resolution of the calorimetric variables, they are sometimes unsuitable for determining thermodynamic parameters due to the high degree of nonreversibility observed in most denaturation reactions at high protein concentrations. Because of the high sensitivity of the calorimeter used in these studies, we have been able to work at moderately low protein concentrations, although the reversibility problem has still not been completely eliminated.

We have found in these studies that the differential power input as a function of temperature is quite reproducible on successive scans of the same sample, *i.e.*, successive scans of a solvent–solvent base line for example, usually agree within 0.0001 cal/ml of sample over the entire temperature range 0–75°. However, when the calorimeter is disassembled and the cells emptied and refilled with seemingly identical samples, the lack of reproducibility is closer to 0.0005 cal over the entire temperature interval. That is, the second heating will trace a line nearly parallel to the first but it is likely to be displaced by the equivalent of 0.0005 cal over the entire temperature interval. This is probably due principally to our inability to determine the actual weights of the samples within the precision of the analytical balance, although other factors may also be important.

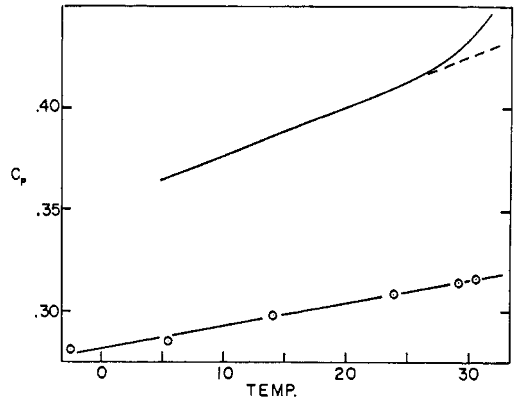

FIGURE 1: Absolute heat capacity of chymotrypsinogen (cal g^{-1} deg^{-1}). Upper solid curve shows the apparent heat capacity of chymotrypsinogen in dilute (1.85%) solution at pH 2 while the lower points show the heat capacity of anhydrous chymotrypsinogen, determined by Hutchens *et al.* (1969).

Because of this, we are able to determine changes in the apparent heat capacity of protein solutions (as temperature is varied) much more accurately than we can determine the absolute values of the heat capacities. This becomes important for studies of denaturation reactions since absolute values of the heat capacity are not necessary and since it is desirable to work with solutions of low protein concentration. For the results reported here on the chymotrypsinogen denaturation, the protein concentrations used were 0.21–0.26%. These concentrations are adequate for measuring changes in the heat capacity with temperature. On the other hand, at these concentrations we observe a large scatter in the apparent values of the absolute heat capacity because of the difficulties described above.

In order to resolve this problem, the absolute heat capacity of native chymotrypsinogen was measured at higher concentrations in a separate experiment. The results are shown in Figure 1 for a pH 2.0 solution and for a protein concentration of 1.85%. The apparent heat capacity of chymotrypsinogen is about 0.4 cal/g deg at low temperature and exhibits a rather strong temperature coefficient at temperatures below that temperature where denaturation is first noticed (*ca.* 25°). Included also in Figure 1 are the results reported by Hutchens *et al.* (1969) for the heat capacity of chymotrypsinogen as a lyophilized powder. Although the heat capacity of anhydrous chymotrypsinogen also exhibits a fairly strong temperature coefficient over the temperature range of interest, the absolute value of the heat capacity is only about 70% as large as the apparent heat capacity of native chymotrypsinogen in dilute solution.

A schematic illustration of data which have been obtained on the chymotrypsinogen denaturation is presented in Figure 2A. This shows the low-temperature segment AB where the experimental heat capacity is that of the native protein, C_p^N; the high-temperature segment CD which

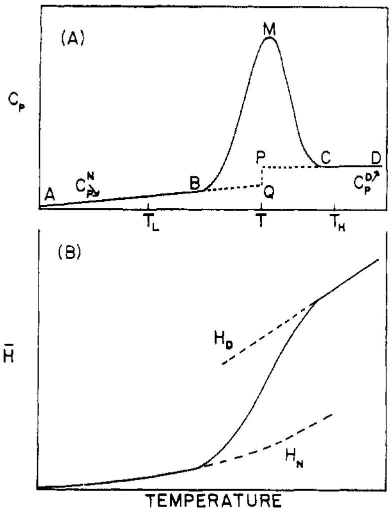

FIGURE 2: Temperature studies. (A) Schematic illustration of the temperature dependence of the heat capacity in the temperature region where the thermal transition occurs. (B) Variation in the enthalpy with temperature in the region of the thermal transition, obtained by integration of the curve in part A. Extrapolated base lines are shown as dashed lines.

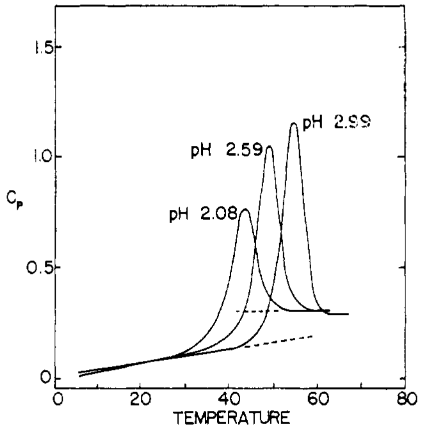

FIGURE 3: Experimentally determined heat capacity profiles for chymotropsinogen at three pH values. The dashed lines represent the extensions of the base lines which were used to estimate thermodynamic parameters. Values of C_p are plotted on an arbitrary absolute scale.

yields the heat capacity of the denatured protein, C_p^D; and the intermediate temperature region where the experimental heat capacity reflects the heat capacity of all states which are present as well as the excess heat necessary to drive the unfolding reaction. The difference in enthalpy between the native and denatured state at temperature, T, is then given by the equation

$$\Delta H^\circ(T) = \int_{T_L}^{T_H} C_p \mathrm{d}T - \int_{T_L}^{T} C_p^N \mathrm{d}T - \int_{T}^{T_H} C_p^D \mathrm{d}T \quad (1)$$

where T_L and T_H are reference temperatures located somewhere below and somewhere above the transition region, respectively. In this case we have chosen the temperature T at the transition temperature (Figure 2A) although, in fact, it could be chosen anywhere on the temperature axis.

It should be noted that, in order to carry out the indicated integrations in eq 1, it is necessary to extrapolate the heat capacity of the native state and of the denatured state over temperature intervals where direct determination is not possible. This is indicated by the segments BQ and CP, respectively, in Figure 2A. It is this extrapolation of high- and low-temperature base lines which probably constitutes the largest error in the determination of both the calorimetric and van't Hoff parameters, since there is always a certain amount of flexibility in fixing these base lines and, particularly, in fixing the slope of the base lines. In the case of chymotrypsinogen, the native base lines invariably displayed a slight positive slope, consistent with that shown in Figure 1, and this was linearly extrapolated into the transition region, as shown in Figure 2A. The base line for denatured chymotrypsinogen was taken as flat in all cases. This choice was consistent with all of the experimental data but the uncertainty in this case is probably greater than for the low-temperature base line, for reasons to be discussed.

With these approximations, the calorimetric value of $\Delta H^\circ(T)$ can be evaluated from eq 1 and will be equal to the area contained within the segments BMCPQ of Figure 2A. The difference between the heat capacity of native and denatured protein, $\Delta C_p(T)$, will then be equal to the length of the segment PQ at the temperature in question. These parameters will measure the differences between the low-temperature native state and the high-temperature denatured state, regardless of the presence or absence of intermediate states in the transition region, since no assumptions need be made about the thermodynamic mechanism of denaturation.

Calorimetric data may also be used to obtain the corresponding two-state estimates of thermodynamic parameters, for comparison with the direct calorimetric parameters. Integration of the experimental heat capacity C_p leads to estimates of the average enthalpy, $\bar{H}$, of the system which varies in a sigmoidal fashion with temperature as indicated by the solid line in Figure 2B. The extrapolation necessary to obtain H_N and H_D in the transition region can be made quite easily from the extrapolated heat capacities shown in Figure 2A, and this leads to the dashed lines shown in Figure 2B.[1] If it is *assumed* that the unfolding reaction progresses as a two-state process, then it will be true that $\bar{H} = xH_D + (1 - x)H_N$, where x indicates the mole fraction of molecules in the denatured state and $1 - x$ the mole fraction in the native state. The two-state equilibrium constant, $x/(1 - x)$, may then be calculated and other two-state thermodynamic parameters can be obtained from the equilibrium constant by the usual van't Hoff analysis.

Some of the calorimetric data which have been obtained for chymotrypsinogen are shown in Figure 3, for three different pH values. These data have been obtained directly from continuous recordings of excess power input as a function of temperature, after smoothing out the short-time thermal noise (period of *ca.* 5 sec) and after correction for the solvent–solvent base line at all temperatures. The

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[1] It should be pointed out that the nonlinearity of the H_N base line in Figure 2B is a direct consequence of the fact that C_p^N varies linearly with temperature (Figure 2A), *i.e.*, the integration of a function with a first-power temperature dependence leads to T^2 dependence.

TABLE I: Estimates of Thermodynamic Parameters for the Chymotrypsinogen Denaturation, Obtained from Analysis of the Calorimetric Data.[a]

pH	Temp (°C)	Direct Calorimetric Anal.		Calorimetric van't Hoff Anal.
		$\Delta H°$ (kcal)	ΔC_p (cal/mole deg)	$\Delta H°$ (kcal)
1.95	40.6	103	4200	94
2.03	42.0	102	3300	98
2.08	42.0	99	3900	98
2.59	48.0	126	3200	126
2.99	53.9	145	2800	133
3.02	54.2	135	2800	134

[a] Estimates pertain to the transition temperature.

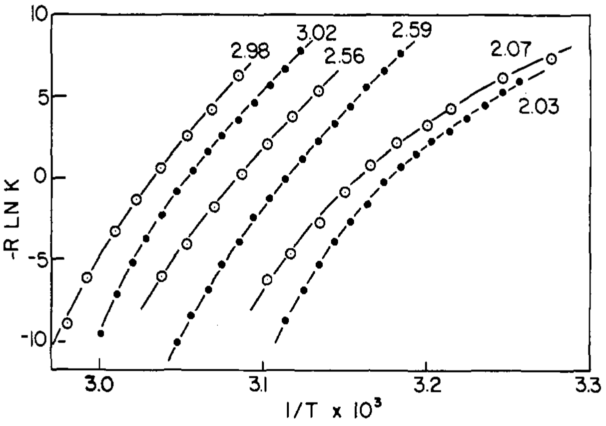

FIGURE 4: Van't Hoff plots for the chymotrypsinogen denaturation. The pH values for each curve are as indicated. Open circles are from the spectrophotometric data of Brandts (1964a), while the filled circles have been determined from the present calorimetric data as indicated in text.

extensions of the high- and low-temperature base lines, used in the calculation of thermodynamic parameters, are shown as the dashed lines in Figure 3.

Each solution examined was heated through the transition region at least twice as a check on reversibility. We have found that the calorimetric reversibility depends on the length of time the denatured protein is exposed to high temperatures. If the first heating is stopped at a temperature where denaturation is just completed, the calorimetric $\Delta H°$ on the second heating can be as much as 95% of that obtained on the first heating. However, in order to accurately estimate the denatured base line, most of these samples were taken about 10° beyond the temperature where denaturation is essentially completed. For all the experiments, the indicated reversibilities ranged from 81 to 95%. These figures probably underestimate the actual reversibility for the first heating since the process of cooling after completion of the heating curve requires nearly twice the amount of time as heating so that more than half of the nonreversibility probably occurred while the sample cooled from high temperatures after termination of the first heating.

It seems likely that the small amount of nonreversibility does not seriously affect the calorimetric values of $\Delta H°$. We have found that the calorimetric $\Delta H°$ determined for a solution of 1.85% protein was indistinguishable from that for a solution of concentration 0.25% (under otherwise similar conditions) even though the reversibility at the higher concentration was less than 50% compared with about 90% at the lower concentration. The principal difference noted between these solutions of differing protein concentration was that, at the high concentration, the indicated ΔC_p was considerably smaller. This could be accounted for if it is assumed that the nonreversibility is a reflection of a time-dependent aggregation process (slightly exothermic) which lowers the value of the high-temperature base line. To what extent these nonreversible effects influence the values of ΔC_p at the lower concentrations cannot be ascertained, although we suspect that it is not too significant.

The calorimetric values of $\Delta H°$ and ΔC_p at the transition temperature are shown in Table I for six separate experiments, corresponding in each case to the first heating. The $\Delta H°$ values show a strong temperature dependence as is to be expected in view of the shape of the calorimetric curves which indicate a large ΔC_p (Figure 3). Over the temperature range from about 42 (pH 2) to 54° (pH 3), $\Delta H°$ increases by about 40% from 100 to 140 kcal per mole.

The calorimetric results also suggest that ΔC_p decreases with increasing temperature. The evidence here comes from two sources. First, for any *single* calorimetric profile our results consistently suggest that the native state heat capacity is an increasing function of temperature while the denatured state heat capacity appears to be much less temperature dependent, as illustrated in the data of Figure 3. Secondly, the trend of ΔC_p values at the transition temperatures for *all* the transitions (Table I) suggest a marked reduction in the magnitude of ΔC_p as the transition temperature is increased by increasing the pH. Although these two independent methods are in agreement regarding the temperature dependence of ΔC_p, the conclusion must still be treated with a certain amount of skepticism since it is conceivable that small amounts of aggregation could make temperature-dependent contributions to the high-temperature base line.

The calorimetric van't Hoff plots (obtained from the two-state analysis of integrated heat-capacity curves, similar to that shown in Figure 2B) for three of the solutions examined in this study are shown in Figure 4. Spectrophotometric van't Hoff plots taken from Brandts (1964a) are also shown for three solutions of similar pH. The lack of correspondence of the transition temperatures for the calorimetric and spectrophotometric curves at the higher pH values are consistent with the much higher ionic strengths for the buffered solutions used in this study, since Brandts has reported a lowering of the transition temperature with increasing ionic strength.

In general, the agreement between the calorimetric and spectrophotometric curves of Figure 4 is as good as can be expected. The curves tend to be nearly parallel at comparable temperatures and exhibit nearly the same curvature. Certain discrepancies can be noted at temperatures far removed from the transition temperatures, but these can probably be attributed to the difficulty in obtaining accurate estimates of the equilibrium constant near the ends of the transition curve. The values of $\Delta H°$ obtained from the calorimetric van't Hoff curves for each of the six samples, at their respective transition temperatures, are listed in Table I. It can be seen that these also agree well with the direct calorimetric estimates obtained for the same samples.

Discussion

There is very little published data on the absolute heat capacity of native proteins. The value of the heat capacity found for native chymotrypsinogen (0.40 cal g^{-1} deg^{-1} at 20°) in dilute solution is in very good agreement with the value of 0.40 reported by Bull and Breeze (1968) for native ovalbumin in dilute solution at 11°. The latter value is somewhat lower than the value of 0.46 reported earlier by Kresheck and Benjamin (1964) for the same protein at 25°. Insofar as we are aware, this is the first instance in which the temperature dependence of the heat capacity has been determined for any native protein in solution. The rather strong positive temperature coefficient observed for native chymotrypsinogen in solution (an increase of 0.7% in C_p with each degree rise in temperature) is approximately the same as that reported for the anhydrous protein (0.6%) over the same temperature interval so it seems possible that the observed temperature dependence arises, in part, from the normal thermal excitation of internal degrees of freedom of the protein, whether it be solvated or anhydrous.

It is difficult to explain the fact that the absolute value of the apparent heat capacity of native chymotrypsinogen in dilute solution is about 30% larger than the value found for anhydrous chymotrypsinogen (and, also, anhydrous insulin) by Hutchens *et al.* (1969) over an equivalent temperature range. This observation is in agreement with the data of Bull and Breeze (1968) who also found that native ovalbumin in dilute solution has a heat capacity which is about 30% larger than that for anhydrous ovalbumin.[2] One could explain the excess heat capacity by postulating that the fully solvated native protein possesses a greater structural lability than does the anhydrous protein, or that the water of solvation of the protein exhibits an anomalously high heat capacity due perhaps to contact with exposed hydrophobic groups of the native protein. Other interpretations may be equally probable, however, since the heat capacity parameter for complex molecules is still only poorly understood.

The principal purpose of this work is to assess the quantitative validity of the two-state mechanism for the chymotrypsinogen denaturation. In doing this, it is well to remember that the two-state mechanism is *always* an approximation for any reaction so that it is prudent to avoid absolute conclusions with regard to whether a reaction "is" or "is not" a two-state process. Furthermore, it seems likely that the quantitative validity of the two-state mechanism will depend upon which thermodynamic parameters are under consideration. For example, in certain cases the two-state approximation may be quantitative for determining changes in first derivatives of the free-energy function (enthalpy, entropy, and volume) but may break down with certain higher order derivatives (heat capacity, temperature dependence of the heat capacity, compressibility, thermal expansion, etc.) since the presence of small concentrations of intermediate states in the transition region and/or small systematic errors unavoidable introduced by improper base-line extrapolations will become increasingly important as successive derivatives of the free-energy function are considered.

There are several sets of data in the literature where estimates of $\Delta H°$ have been made for the chymotrypsinogen denaturation in acid solutions. Unfortunately, solution conditions were slightly different in each case and it can be shown that the small differences in solution conditions will have a significant effect on $\Delta H°$. In the present studies, for example, all solutions below pH 2.5 were unbuffered while those above pH 2.5 were buffered with glycine. In earlier spectroscopic studies, Brandts (1964a) studied the denaturation over the pH range 1–3 using unbuffered solutions (protein concentrations of *ca.* 0.07% or 1.4×10^{-5} M) while Shiao *et al.* (1970) used conditions identical with those of Brandts except that the protein concentrations were only one-half as large.

These differences in conditions are important because the chymotrypsinogen denaturation exhibits a strong pH dependence. It has been shown (Brandts, 1964a) that the pH dependence of $\Delta F°$ (two state) is accurately described (over the pH range 1–3) by

$$\frac{\Delta F°}{T} = \frac{\Delta F_0°}{T} - nR \ln \frac{(1 + a/K_D)}{(1 + a/K_N)} \tag{2}$$

where a is the hydrogen ion activity, $\Delta F_0°$ is a pH-independent free-energy function and where n may be regarded as the *effective number* of carboxyl groups with different protonic dissociation constants in the native state (where the *average* dissociation constant of the n groups is K_N) and in the denatured state (where the *average* dissociation constant is K_D). The experimental data are fit well if the values of n, K_N, and K_D are chosen to be 3.18, 5×10^{-2} M, and 3×10^{-5} M, respectively.

When eq 2 is differentiated with respect to reciprocal temperature to obtain the observable $\Delta H°$, a term arises which depends upon the temperature coefficient of the hydrogen ion activity, *i.e.*

$$\Delta H° = \Delta H°{}^{*} - nR\left[\frac{1}{K_D + a} - \frac{1}{K_N + a}\right]\frac{da}{d1/T} \tag{3}$$

where $\Delta H°$ is the observed value of the enthalpy change, and where $\Delta H°{}^{*}$ is a standard enthalpy change which will not depend significantly on small changes in pH which occur as the temperature is varied. It is the last term on the right of eq 3 which will depend on the temperature coefficient of pH, and which may be different for buffered and unbuffered solutions.

In unbuffered solutions, the binding of protons during denaturation gives rise to a small increase in pH as the denaturation equilibrium is shifted by raising the tempera-

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[2] The value reported for the heat capacity of anhydrous ovalbumin by Kresheck and Benjamin (1964), from a single determination, is about 50% larger than that reported by Bull and Breeze (1968). We have assumed here that the value reported by Bull and Breeze is the more reliable since it involved multiple determinations.

ture, and this tends to make the observed ΔH° smaller than would be observed if the hydrogen ion activity were invariant with temperature. The magnitude of the effect will depend on protein concentration (c in moles l.$^{-1}$) and can be calculated from eq 3 and the relation

$$\left(\frac{\mathrm{d}a}{\mathrm{d}1/T}\right)_{\text{unbuffered}} = \frac{\nu c \Delta H^\circ}{4\ R} \tag{4}$$

where ν is the net difference in protons bound to the native and denatured states at the pH in question (ν can be calculated readily from the values of n, K_{N}, and K_{D}) and where ΔH° is the enthalpy change for denaturation. Equation 4 will be true at the transition temperature and also assumes no temperature dependence of the activity coefficient for protons (Bates, 1964).

In buffered solutions, the temperature coefficient of pH is governed by the heat of ionization of the buffer such that

$$\left(\frac{\mathrm{d}a}{\mathrm{d}1/T}\right)_{\text{buffered}} = \frac{-\Delta H_{\text{ion}}a}{R} \tag{5}$$

The average heat of ionization of glycine will be taken as 500 cal mole^{-1} over the temperature range of interest here (Izaat and Christensen, 1968).

Using eq 3, 4, and 5, values of $\Delta H^{\circ *}$ can be calculated and it is these values which should be directly compared in order to assess the quantitative validity of the two-state mechanism. The corrections which must be added to the observed ΔH° values to obtain $\Delta H^{\circ *}$ range from +9 kcal for the data of Brandts (1964a) at pH 3 to *ca.* −1.5 kcal for the calorimetric data at all pH values where glycine buffer was used. The resulting values of $\Delta H^{\circ *}$ are shown in Figure 5. The dashed line summarizes the spectroscopic data (after applying our corrections) of Shiao *et al.* (1970) which comprises 12 experimental points obtained at transition temperatures ranging from 36 to 54°. The *direct* calorimetric values (filled circles) and the *van't Hoff* calorimetric values (open circles) are also shown, as well as the earlier spectroscopic van't Hoff values of Brandts (triangles). It is seen from this comparison plot that all three sets of van't Hoff data are in good agreement with one another and that the direct calorimetric values are probably experimentally indistinguishable from the van't Hoff values. We conclude from this that the two-state approximation is quantitative for the chymotrypsinogen denaturation (over the range of conditions used in this study) in terms of the enthalpy parameter.

A comparison between van't Hoff and direct calorimetric ΔC_{p} values and between the various estimates of the temperature derivative of ΔC_{p} is more difficult to make because of the greater experimental uncertainties. Our calorimetric values of ΔC_{p} (Table I) for all experiments average out to about 3400 cal deg^{-1} mole^{-1} at an average temperature of 47°. The corrected line in Figure 5 for the spectroscopic data of Shiao *et al.* (1970) leads to a value of 3200 cal mole^{-1} deg^{-1}, using the assumption of these authors that ΔC_{p} is nearly temperature independent. Brandts (1964a,b) originally fit the two-state free-energy values (obtained over a temperature range 20–60°) for chymotrypsinogen denaturation to a power series in absolute temperature, such that a ΔC_{p} value which increased with increasing temperature was obtained upon differentiation of the power series. The ΔC_{p} values which were obtained from his analytical expression vary from about 2400 cal deg^{-1} mole^{-1} (20°) to 4200 cal deg^{-1} mole^{-1} (60°). He later concluded (Brandts, 1969) that the determination of the temperature dependence of ΔC_{p} was probably outside the experimental certainty of his data but that the data were sufficiently accurate to determine the *average value* of ΔC_{p} over the temperature interval in question. This would lead to an estimate of about 3000–3500 cal deg^{-1} mole^{-1} for ΔC_{p} at an average temperature of *ca.* 40°. Thus, the three independent sets of data lead to experimentally identical estimates of the average ΔC_{p} with no obvious discrepancies between the calorimetric and van't Hoff methods. This indicates that the two-state approximation is sufficiently quantitative to permit evaluation of the second derivative of the free-energy function when data are available over a large temperature interval.

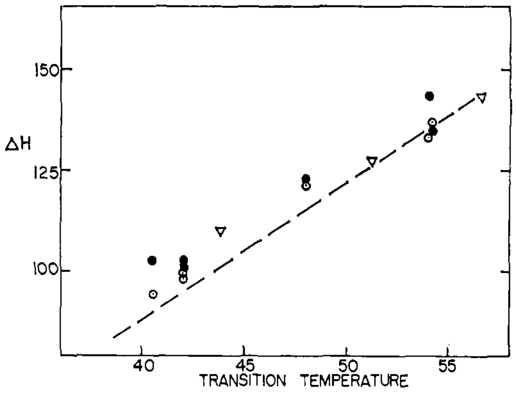

FIGURE 5: Estimates of the enthalpy change for chymotrypsinogen denaturation, after adjustments to correct for the temperature coefficient of pH. The dashed line is from the data of Shiao *et al.* (1970) while the triangles are spectrophotometric van't Hoff values from the data of Brandts (1964a). The direct calorimetric values (filled circles) and the calorimetric van't Hoff values (open circles) from the present study are also included. Values are in kcal mole^{-1}.

This is as far as the comparison of direct calorimetric and van't Hoff parameters can be taken at the present time. It is likely that none of the van't Hoff data now available are sufficiently accurate to permit a reliable evaluation of the third derivative of the free-energy function. Even if data of much higher accuracy are obtained, the problem concerned with extrapolation of base lines will make it exceedingly difficult to evaluate the temperature dependence of ΔC_{p} over the temperature interval available for most reversible denaturation reactions.

The direct calorimetric method offers considerable advantage in determining the temperature dependence of ΔC_{p} since, in this case, it is the heat capacity itself which is the experimental observable so that the first derivative is capable of yielding the same information as can be obtained from the third derivative of spectroscopic and other van't Hoff observables. In the case of chymotrypsinogen, we feel that the present calorimetric data strongly suggest that the temperature coefficient of ΔC_{p} is negative with an order of magnitude of *ca.* 50–100 cal deg^{-2} mole^{-1}. This conclusion must be regarded as somewhat tentative however since the effects of small amounts of irreversibility which arise at high temperatures cannot be adequately determined.

In summary, this analysis supports the earlier suggestion that the two-state approximation can be used to quantitatively determine certain thermodynamic parameters for the chymotrypsinogen denaturation reaction in acid solutions below pH 3. At the current limits of experimental resolution, we find no evidence that the approximation is breaking down. Evidently, the cooperativity of this unfolding reaction is such that the free energy of states of intermediate folding is so unfavorable that their concentrations is the transition region are always considerably smaller than the combined concentrations of native and denatured states. Although drawing this conclusion, we still reject the notion that native and denatured chymotrypsinogen are "states" whose structure may be regarded as being temperature invariant. It seems highly probable that all native and denatured proteins will undergo some thermodynamic reorganization as the temperature is varied. The high degree of accuracy of the two-state approximation would then seem to indicate that the thermal reorganization which does occur for native and denatured chymotrypsinogen is adequately taken into account by a smooth extrapolation of low- and high-temperature base lines into the transition region (Brandts, 1969).

It should be added that very recent calorimetric results of Rodney Biltonen (personal communication) are in substantial agreement with the results presented in this manuscript. His results were obtained by mixing calorimetry, using pH as the variable that induces denaturation. After subtracting off the heat changes associated with the titration of carboxyl groups, he obtains a ΔH of 123 kcal for the thermal denaturation at pH 2.0 and 50° compared with our value of 126 kcal at 48° and pH 2.59. His results suggest a ΔC_p of 2000 cal deg^{-1} mole^{-1} at 50°, which is slightly smaller than the value of 2800 cal deg^{-1} mole^{-1} reported in this study at 54°. He finds a heat capacity for native chymotrypsinogen of 0.36 cal deg^{-1} g^{-1} at 25° and a temperature coefficient of 0.003 cal deg^{-2} g^{-1}, which compare with the present values of 0.41 cal deg^{-1} g^{-1} and 0.003 cal deg^{-2} g^{-1}, respectively. He concludes, as do we, that ΔC_p for denaturation exhibits a negative temperature coefficient.

It still seems likely, as stated earlier (Brandts, 1964, 1969; Brandts and Hunt, 1967) that the large increase in heat capacity during denaturation results primarily from the exposure of hydrophobic side chains to solvent, in agreement with experimental data on small hydrophobic solutes which show that such molecules have heat capacities when dissolved in water which are more than twice as large as the heat capacities when in the pure state or when dissolved in organic solvents. The apparent negative temperature coefficient of ΔC_p found in these studies seems also to be in agreement with this hypothesis. We have observed in calorimetric studies (unpublished data) on small hydrophobic solutes (diethylamine, ethanol, tetrabutylammonium bromide, tetrahydrofuran, and tetraphenylboron) in water over the temperature range from 0 to 85° that the temperature dependence of the apparent molal heat capacities is negative for all solutes over most of the temperature range, as opposed to a positive temperature coefficient which is found for almost all *pure* organic compounds, both solids and liquids. The same trends are seen in the published data of Alexander and Hill (1969) on the heat capacities of solution of the alcohols (methanol through butanol) in water since ΔC_p of solution exhibits a negative temperature coefficient for the large hydrophobic alcohols. If the principal determinant of ΔC_p for protein denaturation is the excess number of exposed hydrophobic groups in the denatured state, it would then be anticipated that the temperature coefficient of ΔC_p would be negative, as we have tentatively concluded.

The paper by Tsong *et al.* (1970) describes experimental studies on the ribonuclease denaturation that are analogous to those presented here for chymotrypsinogen, with the principal difference being that their calorimeter measures directly H *vs.* T, rather than C_p *vs.* T. Consequently, their data are in the form of Figure 2B, which we obtain by integration. In general, our integrated curves for chymotrypsinogen are quite similar to what they have reported for ribonuclease. They also observe, in the H *vs.* T plots, that the low-temperature "base line" has an upward curvature even at temperatures below that where the major denaturation reaction, as defined spectroscopically, begins to occur. As pointed earlier,[1] we regard this upward curvature as the result of a temperature-dependent C_p for the low-temperature state.

In the treatment of their results, Tsong *et al.* have placed primary emphasis upon a method of data analysis which is different from the method used in this paper. Had their method of data analysis been used in the treatment of our data, the agreement between van't Hoff and direct calorimetric estimates of thermodynamic parameters for the chymotrysinogen denaturation would have been very poor. The problem arises due to differing views as to how to treat the *low-temperature* base line since, other than this, the two methods of data analysis are completely equivalent. In their method, Tsong *et al.* have chosen as their low-temperature base line a line of zero slope that intersects the experimental data (referring to Figure 2B) at the lowest temperature where data were obtained (*ca.* 20° in their experiments) corresponding to the $H = 0$ point on the arbitrary enthalpy scale. Their native state base line would then be the coordinate axis in Figure 2B (and also the coordinate axis in Figure 2A). Consequently, their ΔH, value would correspond to the value of H_D at temperatures in the transition region since H_N is defined as zero at all temperatures, whereas our value of ΔH is the difference between H_D and the extrapolated curve labeled H_N in Figure 2B, as discussed earlier. It is readily seen that calorimetric ΔH values obtained by our method of analysis will always be *smaller* than those obtained by their alternative analysis, given the same set of experimental data. The discrepancies between the two methods will tend to become larger as the major transition is shifted further away from the low-temperature starting point as will happen, for example, as the pH is increased. Furthermore, the difference in the choice of base line will tend to have the *opposite* effect on the derived van't Hoff calorimetric ΔH values, *i.e.*, the estimates of the calorimetric van't Hoff values using our low-temperature base lines will tend to be considerably *larger* than those calculated using the method of Tsong *et al.*

The net effect of all this is that in situations where our method might yield good agreement between the values of the direct calorimetric ΔH and the van't Hoff calorimetric ΔH, their method would suggest that the direct calorimetric ΔH is considerably larger than the van't Hoff calorimetric ΔH so that the two methods would lead to opposite conclusions regarding the validity of the two-state mechanism.

It appears to us that the difference of opinion concerning the analysis of calorimetric data has no bearing on the validity of the conclusions reached earlier in this paper, but is concerned exclusively with the fact that our method of data analysis yields thermodynamic parameters for a *different reaction* than does the method emphasized by Tsong *et al.* due to the different way in which the "native" or low-temperature state has been defined. We have, in effect, defined the native state as that macroscopic state which exists exclusively at all temperatures below the beginning of the major unfolding transition, as defined spectroscopically, viscometrically or in terms of other physical observables (Brandts and Lumry, 1963). Any temperature dependence of the heat capacity that occurs in this temperature region then reflects characteristics of that state, whether that temperature dependence is the result of normal thermal excitation of internal degrees of freedom, small changes in conformation resulting from thermal perturbations, or the result of changes in solvent–solvent or solvent–protein interactions. The dynamic properties of this state are then extrapolated into the transition region on the basis of the behavior in the low-temperature region. When this definition is applied consistently to both the high- and low-temperature states, and applied consistently in the case of different physical observables, the method of analysis is self-consistent and we can reasonably ask the question as to whether the transition between these states, *as defined*, obeys the two-state approximation. The answer to this question appears to be in the affirmative for chymotrypsinogen within the restrictions noted earlier. It is important to emphasize again that when "states" are defined in this way, they will invariably be macroscopic in nature and are best thought of as a *distribution* of microscopic states (Hill, 1963; Lumry *et al.*, 1966; Brandts, 1969) that will consist of a large number of occupied energy levels for *all degrees of freedom* including rotational, vibrational, librational, "conformational," and those degrees of freedom associated with solvation. If the transition between macroscopic states *strictly* obeys the two-state approximation, it is to be concluded that no "unique" microscopic states of significant population occur in the transition region, except insofar as these are predicted from the extrapolation of the high- and low-temperature base lines (Brandts, 1969).

The low-temperature base line used by Tsong *et al.* necessitates that their reported calorimetric values refer to the enthalpy change for the transition from native protein at low temperature (*ca.* 20°) to denatured protein at the transition temperature which is clearly a different process than that discussed above, where the enthalpy differences refer to the two states at the *same temperature* insofar as this can be determined by extrapolation.

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