were thermostated, prior to use, in a constant-temperature bath. A 1.50-ml aliquot of the appropriate base solution was transferred to the reaction cell (Pyrocell Manufacturing Co.) in the thermostated cell jacket of the spectrophotometer and allowed to equilibrate thermally for at least 3 min. The reaction was then initiated by rapid injection of 1.00 ml of the substrate solution into the cell from a thermostated syringe. The increase of ultraviolet absorption due to formation of the *N*-methylaniline product (XC_6H_4-$NHCH_3$) *vs.* time was monitored at these wavelengths (X, λ in mμ): *m*-NO_2, 240; *m*-Br, 245; *m*-Cl, 243.5; *p*-Br, 247; *p*-Cl, 246.5; *m*-CH_3O, 239; H, 238; *m*-CH_3, 242; *p*-CH_3, 238; *p*-CH_3O, 300. For all cases except X = *p*-OCH_3, the substrates have no absorption maxima in the 200–320-mμ range and show only a small absorption at the wavelength used for kinetic studies. For X = *p*-OCH_3, there was an interfering substrate absorption maximum at 224 mμ, and therefore the much weaker absorption maximum of the product at 300 mμ was used. The base concentration was in large excess over the substrate concentration so that pseudo-first-order kinetics was observed in all cases. The observed first-order rate constants were calculated from the kinetic data by a linear least-squares treatment of the integrated first-order rate law using a GE 625 computer. Reactions in deuterium oxide were carried out with use of a drybox and serum-capped vessels.

***N*-Methylaniline Acidities.** The pK_a values were determined in aqueous solution by the spectrophotometric method of Willi[22] using a Cary Model 16 spectrophotometer. All measurements were carried out at 25.0 ± 0.1° and at a constant ionic strength of 0.05 *M* maintained with added potassium chloride. Sodium acetate–acetic acid (Fisher Scientific Co., Reagent Grade) buffers and dilute hydrochloric acid solutions were used for the control of pH. Spectral data obtained during the work are given in Table VI.

Acknowledgment. We are happy to thank Mr. David Ebel for his excellent technical work.

Studies of the Chymotrypsinogen Family of Proteins. XI. Heat-Capacity Changes Accompanying Reversible Thermal Unfolding of Proteins[1,2a]

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Abstract: The reversible thermal unfolding processes (transition I) of chymotrypsinogen A, dimethionine sulfoxide chymotrypsinogen A, diphenylcarbamyl-α-chymotrypsin, and ribonuclease A have been quantitatively characterized in the pH interval 2–3.5 using a spectrophotometric van't Hoff method. An attempt is made to evaluate baseline errors and the form of the relationship between heat-capacity change and temperature. Comparisons of results with those obtained using calorimetric data indicate that baseline errors have been minimized. It is found that although the heat-capacity change within error is independent of temperature, the errors are so large as to exclude the use of van't Hoff data in establishing the form of the temperature dependence. Nevertheless an analysis of the data based on temperature-independent heat-capacity changes provides values for the enthalpy change and the average heat-capacity change in good agreement with those obtained calorimetrically. The results are qualitatively consistent with the analysis given by Brandts[3,4] but demonstrate the need for a more accurate heat-capacity expression.

The first reversible thermal unfolding transition, transition I, of globular proteins takes them from the most completely folded state, state A, to the first fully or partially unfolded state, state B.[5] Large, positive, heat-capacity changes accompanying transition I were first reported by Brandts[6,7] in studies of chymotrypsinogen A (CGN) and ribonuclease A (RNase). Similar observations have been reported for α-chymotrypsin and its mono- and dimethionine sulfoxide derivatives by Biltonen and Lumry[8,9] and for myoglobin by Hermans and Acampora.[10] Danforth, *et al.*,[11] and Tsong, *et al.*,[12] measured $\Delta C_p°$ for ribonuclease calorimetrically and found it to be about 2.0 kcal/mol deg in agreement with Brandts' van't Hoff value.[7] Pace and Tanford[13] investigated β-lactoglobulin A in aqueous urea solutions and found $\Delta C_p° = 2.1$ kcal/mol deg. More recently Schwartz, Wadsö, and Biltonen[14] have measured $\Delta C_p°$ calorimetrically at several temperatures using proteins of the chymotrypsinogen A family. Large positive $\Delta C_p°$ values were also observed. Such $\Delta C_p°$ values appear to be a general characteristic of unfolding transitions and have been attributed by Brandts to changes in the interaction of nonpolar polypeptide side chains with water. As a result of the magnitude of $\Delta C_p°$ van't Hoff plots at lower temperatures are very curved. In order to analyze these plots Brandts gave a provisional expres-

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sion for the free energy of unfolding processes, $\Delta F°$, as a function of temperature, salt concentration, and pH. On the basis of previously reported values for the heat capacity of transfer of amino acids from polar organic solvents to water, he assumed $\Delta C_p°$ for proteins to be temperature dependent and chose a polynomial dependence of $\Delta F°$ [6] on T as had been used for fitting the amino acid solubility data. In order to make comparisons between the van't Hoff and calorimetric results as is necessary to determine the nature of the unfolding process and to provide a basis for analysis of calorimetric data, it is essential that the most accurate possible choice of temperature dependence of $\Delta C_p°$ be made. Because of systematic errors the van't Hoff method is less accurate than the calorimetric method although until recently it has been at least as precise. Rapid improvements in calorimetry as applied to protein solutions make it now necessary to examine Brandts' choice of free-energy expression. The following questions have become important. (1) Because of the arbitrariness introduced by the choice of mathematical interpolation, can one obtain accurate heat-capacity information about unfolding reactions of proteins from thermal-equilibrium studies? (2) What empirical equation best describes quantitatively the thermodynamic information about transition I? These questions are considered in this article.

Analysis of data on conformational transitions is simple only if the transitions can be approximated by a two-state model. The physical meaning of this approximation has been discussed by Lumry, *et al.*[15] There is still considerable uncertainty about the validity and limits of this approximation and although there are tests based on van't Hoff or kinetics data which can be used to test the approximation in given instances, the validity of the approximation in any specific case is best tested by comparison of the calorimetric data with the van't Hoff data ("the comparison test") provided that there is no uncertainty in analyzing experimental data in both methods. Transitions I or chymotrypsinogen A[16] (CGN), ribonuclease A[11,12,17] (RNase), α-chymotrypsin[8,14] (α-CT), and myoglobin[10] have been shown to be consistent with the two-state model under some conditions and with some tests. Those of CGN,[16] α-CT,[14] and RNase[11,12,17] in the acid pH region have been satisfactorily established as two-state phenomena by comparison of calorimetric results[11,12] with those obtained by the van't Hoff method.[8,12] The CGN situation has recently been shown by Bolen and Biltonen[18] and Shiao and Sturtevant[19] to be complicated by a pH-dependent process with an effective pK_a of about 2, possibly the disruption of the His-40 to Asp-194 ion pair. When transition I is forced to occur near pH 2, this substrate transition is superimposed on the low-temperature end of transition I. The separation of these two processes has reconciled the calorimetric data of Schwartz, *et al.*,[14] with the data of Jackson and Brandts.[16] A similar situation exists for α-chymotrypsin but the substate transition, possibly the opening of the Ile-16 to Asp-194 ion pair, which has a pK_a value of 3 in moderate ionic strength, has a very small enthalpy change, 4 kcal/mol, and is not detected in most transition I experiments since the errors are usually of this magnitude.[8] Technically transition I for CT can be said to be a three-state process when it occurs near pH 3 and the same thing can be said for CGN near pH 2. The distinction between two-state and multi-state transitions is a quantitative matter susceptible to several kinds of ambiguity.[15] One important example is confusion of substrate transitions with state transitions. The original choice of terms was arbitrary. It was made on the basis of long-range utility but it must be applied with caution.[5,20]

A second type of complication which can develop in an attempt to decide whether or not an unfolding transition is two state has to do with the interpretation of baseline thermal behavior. It can occur in calorimetric studies as well as in van't Hoff studies. Only with certain types of kinetics tests[21] and in studies such as those possible with nmr[22] in which the number of different species can be clearly established at each point along the temperature curve can the baseline problem be bypassed. In RNase A, Tsong, *et al.*,[12] find that the comparison test confirms two-state behavior at lower pH values but the heat uptake in their heat-capacity calorimetry rises with temperature at temperatures below the main transition range. The aggregate heat input before the main transition at pH values above about 2.8 is a significant fraction of the total heat input in the experiment. This heat can be interpreted as due to the heat capacity of state A or to a small, weakly cooperative thermal transition. Tsong and coworkers favor the latter interpretation and its use leads directly to the conclusion that the comparison test may fail for RNase at the higher pH values. However, until significant changes in the characteristics of the A state can be demonstrated to occur during the heating process before the system moves into the temperature range of transition I, there is little utility in definining an additional state.

Even when a baseline controversy of this type does not appear and it is reasonable to interpret its temperature dependence in terms of a single folded or a single unfolded state, the conversion of the measured values of the observable into equilibrium constants requires very accurate extrapolation of the baselines from temperatures outside the transition range into that range.[16] The estimates thus far made for the heat capacity in state A and state B and for the heat-capacity change in transition I have not yet established the form of the temperature dependence but they do suggest that there is little difference among globular proteins in this respect. There are, of course, ample reasons for wanting to know the form of this dependence, but in addition, if there is a single form, the necessary extrapolations of the baselines can be made with confidence and in this way eliminate the remaining uncertainty in the measurement of the enthalpy and entropy changes in transition I. Our study was undertaken to see if van't Hoff data obtained with very high precision could be used to give a good estimate of the form of the tem-

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perature dependence and thus a good estimate of the heat capacities. It easily can be shown that when the state-A and the state-B baselines are equally susceptible to systematic errors, as is usually the case, such errors produce the smallest error in the computed enthalpy change when the computation is made from data obtained at temperatures near that at which the two states are equally populated, *i.e.*, near $\Delta F° = 0$. We have thus varied this temperature, T_0, by increasing pH in successive small increments and calculated $\Delta F°(T)$ at T near T_0 from the resulting van't Hoff plots.

This procedure introduces a pH as well as a temperature dependence. In order to separate these dependencies as well as one can without calorimetry it is necessary to have transition curves at single pH values covering as large a fraction of the total transition as possible, *i.e.*, covering a temperature range which includes as much of the transition range as possible. The reaction must also be strictly reversible under all experimental conditions for which one hopes to calculate $\Delta F°$. For the chymotrypsinogen family it has been found that difficultly reversible aggregation of the unfolded species and the autolytic digestion of proteolytically active forms are the major factors responsible for irreversibility.[23] Aggregation is strongly dependent on temperature, pH, protein concentration, and salt concentration to such an extent that it is not yet possible to obtain transition I data at pH values much greater than 4 except for a few chemically modified members with large changes in charge groups[24] or using rapid heating and cooling experiments.[25] As a result of these restrictions among the group of CGN and CT proteins currently available the dimethionine sulfoxide derivative of CGN (DMSCGN) best satisfies the requirement for single transition curves at constant pH. In addition, CGN, previously studied by Brandts,[6] and diphenylcarbamyl-α-CT (DPC-α-CT) have been used as maximally different members of the chymotrypsinogen family and RNase to provide some information about the variability of fitting parameters within and between families of proteins.

Experimental Section

Materials. Three-times crystallized DBN obtained from Worthington Biochemical Corporation was used without further purification. The purification procedure suggested by Yapel[26,27] has no effect on the results with CGN presented in this study. We shall discuss this point in the Results. Five-times crystallized ribonuclease A was purchased from Nutritional Biochemicals Corporation. In order to obtain the pure ribonuclease-A fraction, the method of Hirs, *et al.*,[28] was employed. The separated ribonuclease-A solution was dialyzed against 0.001 *M* HCl and then used without further treatment. The preparation and characterization of DMSCGN and DPC-α-CT are discussed elsewhere.[24] Other reagents were all reagent grade and were used without further purification.

Instruments. The advancement of the unfolding reactions of the proteins was followed on a Cary-14 spectrophotometer by measuring the near-ultraviolet difference spectra produced during the unfolding processes. A 0–0.1 OD slide wire was used for all experiments. For each measurement, a scan was made from 330 to 270 nm but the final measurements of optical density were made at 293 nm for the CGN family of proteins and 287 nm for RNase. In the long-wavelength region (310–330 nm) the protein solution is transparent and the long-wavelength part of the trace could be used as the zero baseline for each measurement. In this way errors arising from drift of the spectrophotometer were eliminated.

In order to improve the precision in the measurements of optical density change at a specific wavelength the scan was stopped at that wavelength for about 1 min so that an average (average over visual noise level) value of optical density change could be obtained from the steady trace of the recording pen. The precision in measuring optical density change in this way was $\pm 5 \times 10^{-4}$ OD unit which was the noise level of the Cary 14 spectrophotometer with the 0.1 slide wire.

For the purpose of achieving precise temperature control, well-insulated chambers were designed for the reference and the sample compartments of the spectrophotometer. A pair of quartz windows was installed in the light path on each chamber to establish temperature uniformity around the optical cell. It was found that without these windows, serious temperature gradients were introduced in the optical cell. For the reference thermostat, a large-capacity water bath was used to maintain the reference cell near 10°. This temperature was chosen because the rate of spectral change as a function of temperature for all the protein systems studied was observed to be smallest at or near this temperature so that as a result fluctuations of temperature in the reference cell caused minimum error in optical density measurements. The water bath employed in regulating the temperature of the sample thermostat was purchased from Forma Scientific Inc. (Model 2095-2). The stability of temperature achieved by this bath and the thermostat was in general better than $\pm 0.05°$. A Hewlett-Packard Model DY-2801A quartz thermometer with digital print-out was used to measure the temperature in the sample cell. The temperature probe, Model 2850, was immersed in the protein solution and an air-tight Teflon cap was attached to the probe in order to fix the probe position and also to prevent the protein solution from evaporation. In order to avoid any possible chemical complications from the direct contact of the stainless-steel probe with the protein solution, a thin layer of acrylic plastic was applied to the surface of the probe. The quartz thermometer together with the temperature probe was calibrated to 0.01° absolute accuracy. Since the temperature measurements were recorded at the instants when optical density changes were recorded, the precision in temperature measurement at each point was about the same as the accuracy of the quartz thermometer ($\pm 0.01°$).

Procedures Used in Obtaining Thermal Unfolding Data. In a typical experiment the protein solution was made by dissolving approximately 7 mg of protein (about 18 mg of RNase) in 20 ml of glass-distilled water. HCl (1 *N*) and 2 *M* KCl were used to adjust pH and chloride ion concentration of the protein solution. The solution was filtered and the protein concentration was determined spectrophotometrically on a Beckman DU spectrophotometer, there being no need for high precision in the determination of this quantity. The following extinction coefficients were used: for the CGN family of proteins, $\epsilon_{280} = 2.00$ l. g^{-1} cm^{-1}, and for RNase, $\epsilon_{278} = 0.714$ l. g^{-1} cm^{-1}. The protein solution was introduced into the sample and the reference cell and a zero baseline was established by scanning from 330 to 270 nm with both sample and reference temperature the same. The temperature of the sample cell was then varied and the optical density change at 293 or 287 nm was measured after thermal equilibrium of the sample cell was attained (*i.e.*, after the optical density change had reached a steady value). The measurement of optical density change at a specified wavelength as a function of temperature could either be carried out manually or automatically. The automatic device, which could be programmed to carry out an entire experiment including the reversibility check, is described elsewhere together with the detailed experimental procedure, the method of analysis of data, and the statistical treatment.[24,29]

Results

Reversibility. Two tests were used in this study to ensure reversibility of transition I: (1) data were collected during both halves of the temperature cycle (under such circumstances, 100% reversibility was con-

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firmed) or (2) after transition I was complete, the sample bath was cooled down to the same temperature as the reference cell and a difference spectrum was measured. In test 2 if the difference in optical density at a specified wavelength was less than 2% of the magnitude observed when the protein solution in the sample cell was totally unfolded, the process was considered to be totally reversible. Most of the transition I data reported here are from completely reversible experiments. Under some conditions, the reversibility of transition I is only about 90% according to test 2 and these experiments are indicated in Tables I, II, and III. It was found later that even under this circumstance, better than 95% reversibility could be achieved if the cooling of the sample cell was carried out slowly. Hence, even these experiments were very nearly reversible.

Table I. $\Delta H°$ Values of DMSCGN at T_0 under Various Conditions

T_0, °K	$\Delta H°$, kcal/mol	pH	[Cl⁻], M
297.7	34 ± 4	2.0	0.01
306.0	52 ± 4	2.32	0.01
310.4	61 ± 4	2.60	0.01
316.7	70 ± 4	3.00	0.01
322.8[a]	84 ± 6	3.55	0.01
323.2[a]	88 ± 6	3.65	0.01
318.7	78 ± 5	3.08	0.01
313.7	68 ± 4	2.78	0.01
318.4	75 ± 5	3.10	0.01
303.0	42 ± 4	2.20	0.01
305.3	49 ± 4	2.31	0.01
313.8	68 ± 4	2.59	0.005
314.8	72 ± 4	2.59	0.003
311.1	65 ± 4	2.65	0.01
296.9	31 ± 4	1.90	0.013

[a] Under these conditions, the unfolding process was not 100% reversible.

Table II. $\Delta H°$ Values of CGN at T_0 under Various Conditions

T_0, °K	$\Delta H°$, kcal/mol	pH	[Cl⁻], M
325.3	127 ± 8	2.8	0.01
328.1	140 ± 9	3.0	0.01
314.7	92 ± 4	2.0	0.01
317.5	102 ± 4	2.2	0.01
320.0	109 ± 5	2.4	0.01
322.7	116 ± 5	2.6	0.01
331.2[a]	146 ± 9	3.2	0.01
333.7[a]	156 ± 9	3.6	0.01
309.6	79 ± 4	1.6	0.025
313.5	88 ± 4	2.0	0.05
313.4	90 ± 4	2.0	0.03
329.4[a]	140 ± 9	3.0	0.001

[a] Under these conditions, the unfolding process was not 100% reversible.

It should be noted that the complete reversibility observed in most of the transition I processes investigated in this study is a consequence of low protein concentration used in all the experiments (approximately 0.05% for the CGN family of proteins and 0.15% for RNase).

Unfolding Free Energy as a Function of Temperature. Equilibrium constants (K) at various points in the transition region were calculated, on the basis of the two-state model for the unfolding process, by

$$K(T) = \frac{\Delta\epsilon(T) - \Delta\epsilon_A(T)}{\Delta\epsilon_B(T) - \Delta\epsilon(T)} \quad (1)$$

eq 1 where $\Delta\epsilon(T)$ = optical density difference at temperature T, $\Delta\epsilon_A(T)$ = optical density difference at T when the system is in the native state, A, $\Delta\epsilon_B(T)$ = optical density difference at T when the system is in the unfolded state, B. The major problem in eliminat-

$$\Delta F°(T) = -RT \ln K(T) \quad (2)$$

ing systematic errors in this procedure lies in the determination of $\Delta\epsilon_A$ and $\Delta\epsilon_B$ as functions of temperature. With proteins of the CGN family Brandts[6] and Biltonen and Lumry[8] found a complicating spectral perturbation due to changes in charge on these proteins. These authors provided a means for making a suitable correction for this effect but the only available procedure for obtaining $\Delta\epsilon_A$ and $\Delta\epsilon_B$ in the temperature interval of the transition is to vary pH or salt composition or both so as to shift the temperature interval of the transition up or down, each shift providing a segment of either the $\Delta\epsilon_A$ or $\Delta\epsilon_B$ baseline. Although this procedure, which does not, of course, provide the complete baselines, has been used in the analysis of transition I data for CGN, α-CT, and dimethionine sulfoxide-α-CT apparently with success, it was found to be unreliable in this work. In several instances very large errors appear to be introduced by this method. In particular the comparison of enthalpy changes at different pH values but constant temperature, which would only be made between the foot and the top of two different transition curves where errors due to baseline uncertainties are largest gave large pH dependencies of $\Delta H°$. This observation is inconsistent with results reported by others for proteins of the CGN family and led to the method already discussed in which only $\Delta H°(T_0)$ is determined. This method gave reasonable results with a high degree of consistency among the proteins of the chymotrypsinogen family[24] as will be shown in a subsequent paper of this series. The values obtained by this procedure are given in Tables I, II, III, and IV. The errors indicated in these tables are the estimated uncertainties in measuring the van't Hoff slopes and are

Table III. $\Delta H°$ Values of DPC-α-CT at T_0 under Various Conditions

T_0, °K	$H°$, kcal/mol	pH	[Cl⁻], M
304.2	84 ± 4	2.0	0.01
298.2	60 ± 4	1.6	0.025
319.8	142 ± 6	2.9	0.01
305.3	86 ± 4	2.05	0.01
309.0	100 ± 4	2.2	0.01
312.3	115 ± 5	2.4	0.01
315.2	126 ± 5	2.6	0.01
313.9	119 ± 5	2.5	0.01
306.8	92 ± 4	2.15	0.01
296.9	50 ± 4	1.5	0.032
292.4	27 ± 4	1.3	0.05
325.5[a]	166 ± 6	3.2	0.01
322.3	150 ± 6	3.0	0.01
319.9	142 ± 6	2.9	0.01

[a] Under these conditions, the unfolding process was not 100% reversible.

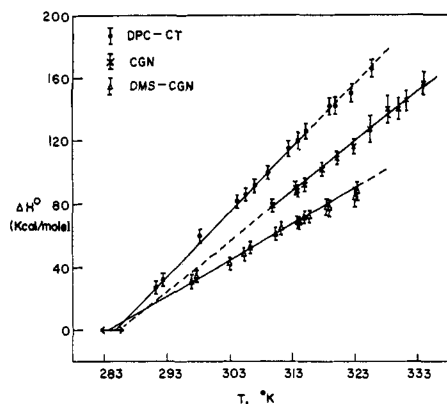

Figure 1. The temperature dependence of ΔH° of unfolding for various protein systems. See text for detailed discussion on the significance of this figure.

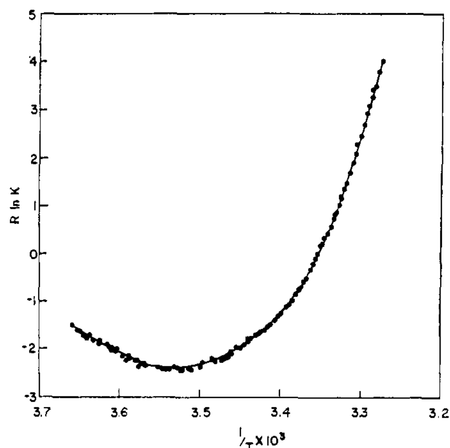

Figure 2. The van't Hoff plot for the thermal unfolding process of DMSCGN at pH 2.

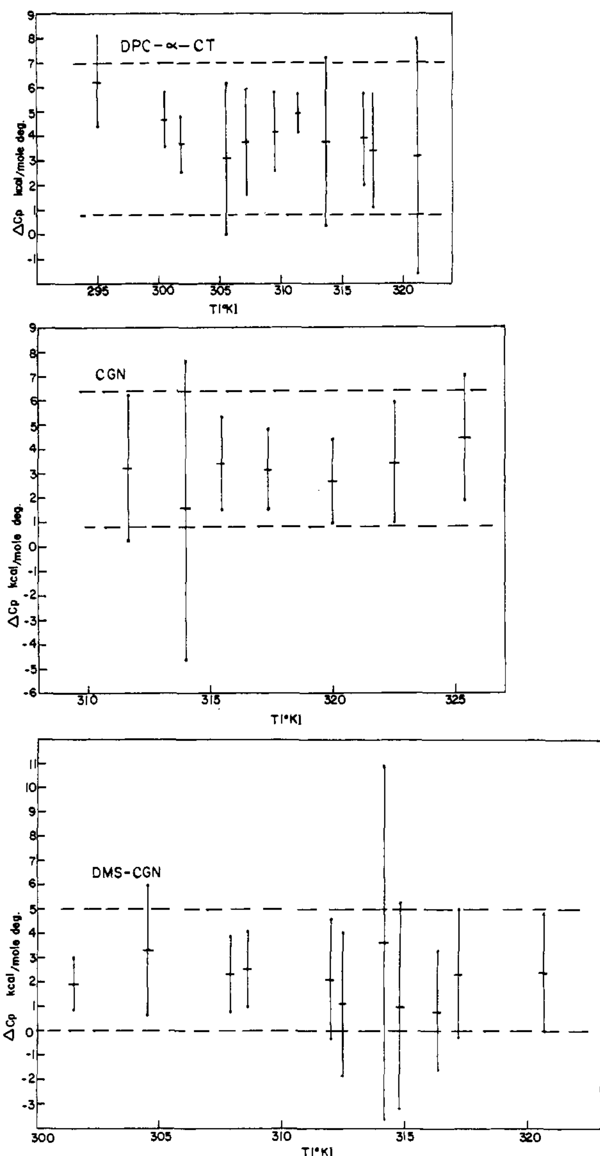

Figure 3. The temperature dependence of ΔC_p° of unfolding of DPC-α-CT, CGN, and DMSCGN. See text for discussion of the preparation and significance of this figure.

of the same order as the systematic errors due to uncertainty in baseline extrapolation. The T_0 values could be determined with a precision no worse than ± 0.2 which is the baseline error and will be neglected in further error discussions.

Table IV. ΔH° Values of RNase at T_0 under Various Conditions

T_0, °K	ΔH°, kcal/mol	pH	[Cl⁻], M
299.9	65 ± 3	2.0	0.01
304.1	70 ± 3	2.2	0.01
308.2	75 ± 3	2.4	0.01
311.4	80 ± 3	2.6	0.01
314.5	83 ± 3	2.8	0.01

Preparative procedures for all the proteins but CGN were such as to remove contaminants. Conventional commercial preparations of CGN may contain contaminants of the type found by Yapel, *et al.*,[26] in preparations of chymotrypsin. CGN used without additional treatment was compared in its transition I behavior with material treated by the procedure of Yapel, *et al.*[27] At pH 2 and 0.01 M chloride ion concentration the specially treated material gave T_0 = 314.7°K and ΔH° = 94 kcal/mol. These values are in excellent agreement with those obtained with untreated material (Table II) and show that the contaminants if present have no effect on transition I for this protein.

Since ΔH° values reported in Tables I–IV were determined using unbuffered solutions, a small correction arising from the increase in pH during transition I should be applied to obtain the true ΔH values at a constant pH. The correction method is described by Jackson and Brandts.[16] For CGN, this correction produces an increase in the ΔH° value of about 3% at pH 3. This correction is small relative to our estimates of error and can be ignored. Our ΔH° values may be a bit low at higher pH values but the correction is negligible near pH 2.[30]

Analysis of Data for CGN, DMSCGN, and DPC-α-CT. The ΔH° values and their errors shown in Tables I, II, and III are plotted in Figure 1. The double headed arrow in this figure indicates the tem-

(30) W. Jackson and J. Brandts[16] calculated the correction and applied it to CGN data of this paper. The correction is within our experimental error but has the effect of raising all our ΔH° values by 2–3 kcal/mol.

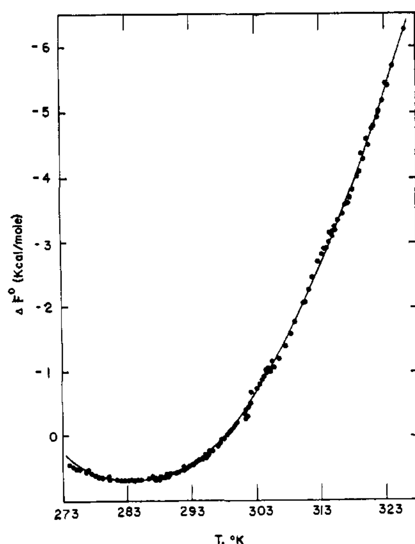

Figure 4. The temperature dependence of ΔF° of DMSCGN corrected to pH 2 and 0.01 M [Cl⁻]. The curve represents the calculated ΔF° values as a function of temperature according to the analytical method discussed in the text.

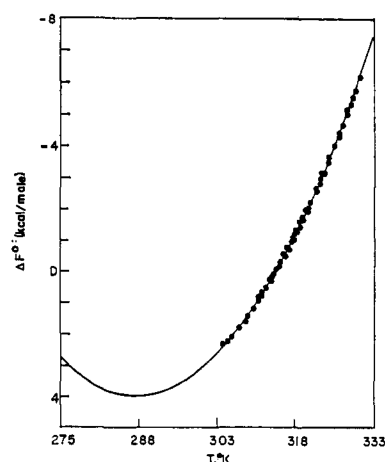

Figure 5. The temperature dependence of ΔF° of CGN corrected to pH 2 and 0.01 M [Cl⁻]. The curve represents the calculated ΔF° values as a function of temperature according to the analytical method discussed in the text.

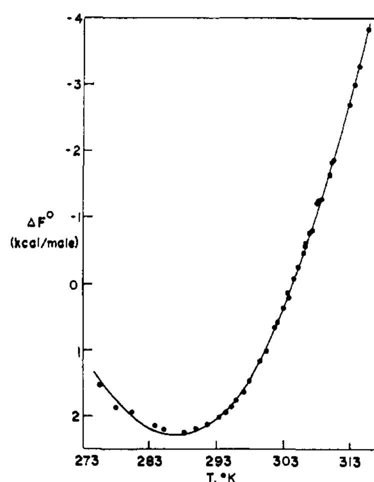

Figure 6. The temperature dependence of ΔF° of DPC-α-CT corrected to pH 2 and 0.01 M [Cl⁻]. The curve represents the calculated ΔF° values as a function of temperature according to the analytical method discussed in the text.

perature region in which ΔH° goes to zero for DMS-CGN, DPC-α-CT. An example of this behavior is given in the van't Hoff plot for DMSCGN at pH 2 (Figure 2). The results in Figure 1 show that for each protein system, ΔH° increases rapidly as T_0 increases suggesting that ΔC_p° for the unfolding reaction is positive and large. To establish this fact it is necessary to know the dependence of ΔH° on pH at constant temperature and salt composition. For CGN Brandts[6] found that ΔH° was independent of pH and chloride ion concentration and Biltonen and Lumry[8,9] found the same independence with α-CT and DMS-α-CT. Brandts found a small pH dependence of ΔH° with RNase which we confirm. RNase thus required a different procedure in interpretation of the data.

Based on the findings of Brandts[6] and those of Biltonen and Lumry,[8,9] it is reasonable to assume that for the CGN family of proteins, the effects of pH and chloride ion concentration on ΔH° are negligible compared to that of temperatue. Figure 1 demonstrates that within the errors ΔH° can be expressed as a linear function of temperature up to the highest experimental temperature. However, the random errors and the possible systematic errors make it impossible to establish the linearity of this plot. We have examined the data for a nonlinear trend by calculating the heat capacity changes between alternate pairs of points for CGN, DPC-α-CT, and DMSCGN. The results plotted in Figure 3 show that there is no detectable nonlinear trend in ΔC_p° as a function of temperature except perhaps for DPC-α-CT at lower temperatures. However, if the dashed lines in Figure 3 which are conservative upper and lower estimates of error are used, there is an uncertainty in that quantity such that if we write $\Delta C_p^\circ = a + bT$, b can be as large as ± 0.25 kcal/mol-deg deg. Although we will interpret this figure as indicating that ΔC_p° is independent of pH and temperature since by so doing the enthalpy changes prove to be in good agreement with most values obtained by others (see Discussion), it may prove necessary in time to examine the alternative interpretation of Figure 1 which is that ΔH° is dependent on both temperature and pH but quite accidentally these two dependencies compensate each other. We will thus avoid an error often made in this type of work by including the raw data from which ΔF° values can be plotted as a function of temperature at a given experimental condition. These data are given later in Table VI.

On the assumption that ΔC_p° is independent of T (as well as of pH and chloride ion concentration in the case of the CGN family proteins), the relevant expressions for $\Delta H^\circ(T)$, $\Delta S^\circ(T)$, and $\Delta F^\circ(T)$ can be written immediately in terms of a constant, $\gamma = \Delta C_p$, a reference temperature, T_r, and values ΔH° and ΔS° at $T = T_r$, ΔH_r° and ΔS_r°, respectively (eq 3–5). We may now

$$\Delta H^\circ(T) = \Delta H_r^\circ + \gamma(T - T_r) \quad (3)$$

$$\Delta S^\circ(T) = \Delta S_r^\circ + \gamma \ln T/T_r \quad (4)$$

$$\Delta F^\circ = \Delta H_r^\circ - T\Delta S_r^\circ + \gamma(T - T_r - T \ln T/T_r) \quad (5)$$

use these equations, particularly eq 5, to fit the ΔF° data points collected near T_0 and thus to provide the best values for the fitting parameters obtainable from the original van't Hoff data. The data points shown in Figures 4, 5, and 6 consist of ΔF° values obtained at

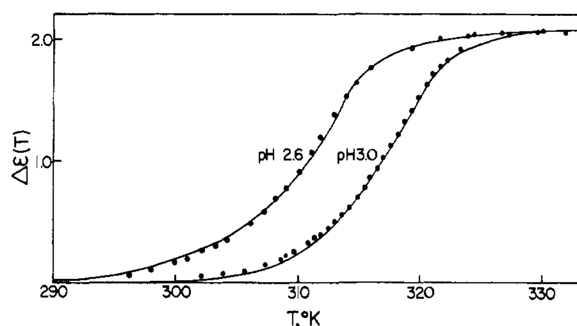

Figure 7. Comparison of the calculated values of $\Delta\epsilon(T)$ of DMS-CGN at pH 2.0 and 3.0 with the experimentally observed data. The solid lines are calculated values of $\Delta\epsilon(T)$. The points are the experimentally observed data. The estimated errors in determining $\Delta\epsilon(T)$ are about the size of the points. See text for more detailed discussion.

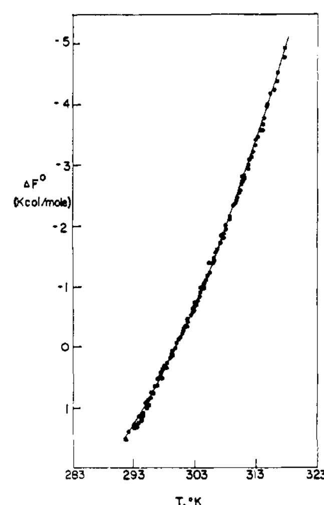

Figure 8. The temperature dependence of $\Delta F°$ of unfolding of RNase corrected to pH 2 and 0.01 M [Cl$^-$]. The curve represents the calculated $\Delta F°$ values as a function of temperature according to the analytical method described in the text.

different pH values but corrected to pH 2 assuming that $\Delta H°$ is independent of pH. The procedure for carrrying out such a correction was described by Brandts.[6] The curves shown in these plots are computed $\Delta F°$ values using eq 5. Only data obtained in completely reversible experiments have been used in preparing these figures. The fitting parameters obtained in this treatment are given in Table V together with their estimated

Table V. Thermodynamic Changes in Transition I for Various Proteins at pH 2.0 and 0.01 M Cl$^-$

Protein systems	T_0, °K	$\Delta H°$ at T_0, kcal/mol	$\gamma = \Delta C_p°$, kcal/mol deg	Estimated error in $\Delta C_p°$,[a] kcal/mol deg
DPC-α-CT	304.1	79.0	4.2	±0.3
DMSCGN	297.7	33.8	2.3	±0.2
CGN	314.5	93.0	3.2	±0.4
RNase	299.9	66.0	2.0	±0.2

[a] The estimated error for RNase is the standard error in the coefficient γ of eq 5; the estimated errors for DMSCGN, CGN, and DPC-α-CT are the uncertainties in measuring the slopes of Figure 1, etc.

errors. The $\Delta H°$ values are calculated for $T_r = T_0$. T_0 is an appropriate reference temperature since although the choice of reference temperature is arbitrary, in practice it should be chosen at the point where the functional relationship between $\Delta F°$ and T is most accurately known. In this work this choice is dictated by the same considerations applying to the choice of $\Delta H°$ values in our attempt to obtain the functional dependence of $\Delta C_p°$ on T.

The fitting parameters $\Delta H°(T_0)$, $\Delta S°(T_0)$, and T_0 obtained by fitting all the data for a given protein in a single operation should be consistent with the eye estimates of $\Delta H°(T_0)$ and T_0 obtained from the individual van't Hoff plots provided only the assumptions that $\Delta C_p°$ is independent of T and that $\Delta H°$ is independent of pH and chloride ion concentrations are at least as precise as our data. On comparison of the best-fit values of $\Delta H°(T_0)$ from Table V with the appropriate values in Tables I, II, or III, we see that only in the case of DPC-α-CT is there a significant error and then only with respect to one standard deviation. Furthermore, since $\Delta H°(T_0)/\Delta S°(T_0) = T_0$, the values of T_0 in Table V can be compared with the T_0 values at pH 2.0 in Tables I, II, or III to demonstrate that the visual determination of T_0 agrees within 0.2 with the best fit value.

The second test for the validity of the interpretation of ΔC_p we have made is more drastic. Using $\Delta H°$ and $\Delta C_p°$ values obtained for pH 2.0 (Table V) and the visually estimated T_0 values we have back calculated to obtain $\Delta\epsilon(T)$ as a function of temperature for DMS-CGN at pH 2.6 and 3.0. The results are shown as the solid curves in Figure 7. It can be seen that the fit is not good especially at pH 3. The deviations are systematic and indicate either errors in our baselines, particularly the state A baseline, or a failure of one or more of our assumptions about $\Delta C_p°$. Jackson and Brandts[16] suggest that $\Delta C_p°$ decreases with rising temperature because $C_{p,A}°$ is a rising function of temperature and $C_{p,B}°$ is independent of temperature. They estimate that $\Delta C_p°$ decreases with a thermal coefficient of 50–100 cal/mol-deg deg, a deviation well inside the range of error shown in Figure 3. Unfortunately their heat-capacity calorimetry does not provide the form of the dependence of $\Delta C_p°$ on temperature other than the indication that it is a decreasing function. This situation together with the possibility that our data still contain baseline errors makes it unrealistic to use those data in further attempts to determine the temperature coefficient of $\Delta C_p°$. On the other hand our values for $\Delta C_p°$ can be considered to be averages over the temperature ranges of our experiments. For CGN our value of 3.2 ± 0.4 kcal/mol-deg at an average temperature of 45° can be compared with the average of Jackson and Brandts, 3.4 kcal/mol at 47°.

Analysis of Data from RNase. With this protein, the state A and state B baselines could be obtained with considerable accuracy for the following reasons. (1) The temperature of maximum stability lies in the range from −5 to 0°[4] so that there are no complications arising from the unfolding of this protein at low temperatures. (2) The unfolded species of this protein appear to resist aggregation at pH values between 2 and 2.8 so that measurements of $\Delta\epsilon$ can be made over a wide temperature range. This is fortunate since in con-

firmation the finding of Brandts and Hunt[17] ΔH° values were found to be pH dependent. To treat this problem, ΔF° values of RNase were plotted as a function of temperature at various pH values and smooth lines were drawn through the points at each pH. At a given temperature, the difference in ΔF°, $\delta\Delta F^\circ$, between two adjacent pH values was then estimated. When this difference was examined as a function of temperature in the temperature region where overlapping measurements between two pH values were possible, it was found that $\delta\Delta F^\circ$ was linearly dependent on temperature which means that the difference in ΔF° due to pH can be represented by the equation $\delta\Delta F^\circ = \delta\Delta H^\circ - T\delta\Delta S^\circ$ in which $\delta\Delta H^\circ$ and $\delta\Delta S^\circ$ are both temperature independent. In other words, the pH dependence of ΔF° has no heat-capacity contribution. This result is equivalent to Brandts' observation that $\Delta C_p{}^\circ$ for RNase is pH independent. Since $\delta\Delta F^\circ$ is linearly dependent on temperature, the plot of $\delta\Delta F^\circ$ *vs.* T between two adjacent pH values establishes the empirical relationship between $\delta\Delta F^\circ$, pH, and T and therefore ΔF° can be corrected to any specified pH by means of this relationship. The results presented in Figure 6 were corrected to pH 2 by this method.

Using the same empirical relationship, it was possible to estimate the pH dependence of ΔH° for RNase by the van't Hoff method. It was found that ΔH° at pH 2.8 minus ΔH° at pH 2.0 was 8 $\pm$ 2 kcal/mol at all experimental temperatures. Brandts and Hunt using Brandts' original expression to get a single transition curve at each individual pH value found that ΔH° at pH 2.77 minus ΔH° at pH 2.1 was 6 kcal/mol independent of temperature. Thus, estimates of the pH dependence of ΔH° of RNase by the two independent methods agree well.

ΔF° values for RNase corrected to pH 2 are shown in Figure 8 and again the curve was calculated using eq 5. The fitting parameters are given in Table V and can be compared with the visually estimated values for this protein. The agreement is very good. The systematic errors in the RNase baselines are thought to be small so that the standard error in γ given in Table V should be a good estimate of the accuracy of $\Delta C_p{}^\circ$.

An important point should be mentioned about the calculation of $\Delta C_p{}^\circ$ of RNase. Since ΔH° of this protein is pH dependent, the slope in the plot of ΔH° *vs.* T_0 using the data tabulated in Table IV cannot be used to determine $\Delta C_p{}^\circ$ values. For the same reason, using the parameters determined at pH 2, eq 4 cannot be used to predict the correct "apparent ΔH° values" determined at T_0 to other pH values unless the pH correction for ΔH° is introduced. For example, according to eq 4 and using the ΔH° value determined at pH 2, ΔH° at 314.5°K, which is the T_0 value of RNase at pH 2.8, should be 94 kcal/mol which is 10 kcal/mol higher than the apparent ΔH° value determined at pH 2.8. Since ΔH° of RNase at pH 2.8 minus ΔH° at pH 2.0 equals $\pm$8 kcal/mol, it should be understood that the difference of 10 kcal/mol is simply a consequence of the pH dependence of ΔH° for this protein.

Discussion

Comparison of Experimental Data. RNase. The van't Hoff data for transition I of RNase in water have been analyzed by Brandts and Hunt.[17] At pH 2.5, the value of ΔH° of RNase at 36° was 75 kcal/mol. Our result (see Table IV) at the same conditions was 77 $\pm$ 3 kcal/mol. The comparison of the $\Delta C_p{}^\circ$ value for RNase obtained in this analysis with that calculated by Brandts and Hunt is not straightforward since the assumptions about the temperature dependence of $\Delta C_p{}^\circ$ in the two analyses are different. They used Brandts' original expression, eq 6, and found the value

$$F = E + FT + GT^2(1 + PT) \qquad (6)$$

of $\Delta C_p{}^\circ$ for RNase varied from 1.7 kcal/mol deg at 20° to 2.4 kcal/mol deg at 45°. Our analysis gave 2.0 $\pm$ 0.2 kcal/mol deg for all temperatures but in view of the development in the previous section this value should be considered to be an average value at 34°.

Transition I of RNase at pH 2.8 was investigated by Danforth, *et al.*,[11] calorimetrically. The values of $\Delta C_p{}^\circ$ measured by them were 1.93, 2.74, 2.86, 2.03, and 2.10 kcal/mol deg. Recent calorimetric measurements on RNase by Tsong, *et al.*,[12] have been interpreted as indicating that the $\Delta C_p{}^\circ$ value is 2.0 $\pm$ 0.1 kcal/mol independent of temperature. The calorimetric value of ΔH° for RNase at 44° was 86 $\pm$ 4 kcal/mol according to Danforth, *et al.*, and their value of T_0 at pH 2.8 was 44°. The latter value is 2.7° too high compared to our results (see Table IV). This discrepancy in T_0 values can be explained by the fact that about 2 mol of protons are taken up by 1 mol of protein in transition 1 of RNase at pH 3 so that at the protein concentration of 1.5% used by Danforth, *et al.*, the hydrogen ion concentration of the protein solution changes throughout the transition region since the protein solution was not strongly buffered. A simple calculation shows that in the presence of 0.01 *M* glycine buffer (which was used in the experiments of Danforth, *et al.*, according to Krakauer[31]), the pH value of a 1.5% RNase solution will change from 2.8 to 3.2 as the protein system is changed from state A to state B. Since the T_0 value of RNase increases as pH increases, the pH change will give an apparent T_0 considerably higher than the T_0 value we have observed under conditions of constant pH. It can be easily shown that at a protein concentration of 0.15% which has been used in our experiments, the pH change due to the absorption of protons by unfolded species of RNase is not significant at pH 2.8.

Since the T_0 value of RNase at pH 2.8 determined in our study is 41.3° which does not agree with that determined by Danforth, *et al.*, a direct comparison of ΔH° at T_0 between these two sets of data is not possible. However, the value of ΔH° measured by Danforth, *et al.*, can be corrected to 41.3° using the $\Delta C_p{}^\circ$ value. The corrected value is 81 $\pm$ 4 kcal/mol which is not inconsistent with our van't Hoff value (84 $\pm$ 3 kcal/mol).

CGN. Brandts, using a spectrophotometric van't Hoff method, found T_0 at pH 2 to be 41.4°. Jackson and Brandts, using calorimetric data for direct heats and for van't Hoff analysis, found the same T_0 which is also that found by us (Table II). Brandts' ΔH at this temperature was 90 kcal/mol; Jackson and Brandts found 96 kcal/mol with van't Hoff analysis and 102 kcal/mol by direct measurement. Our value is 92 $\pm$ 4 kcal/mol

(31) H. Krakauer, personal communication.

Table VI. Free-Energy Data for Transition I of Various Protein Systems

CGN

Temp, °K	$\Delta F°$, cal/mol	Temp, °K	$\Delta F°$, cal/mol
1. pH = 2.8; [Cl⁻] = 0.01 *M*		2. pH = 3.0; [Cl⁻] = 0.01 *M*	
323.22	959	325.59	934
324.02	701	326.42	605
324.82	454	327.22	356
325.63	127	328.06	25
326.47	−187	328.83	−299
327.28	−538	392.63	−694
328.05	−974	330.46	−1,096
328.89	−1,351	331.26	−1,602
3. pH = 2.00; [Cl⁻] = 0.01 *M*		4. pH = 2.20; [Cl⁻] = 0.01 *M*	
311.12	872	313.67	926
311.69	714	314.50	760
312.54	531	315.32	589
313.33	316	316.12	368
314.16	98	316.93	105
315.11	−142	317.77	−137
316.03	−450	318.55	−398
316.85	−718	319.35	−707
317.62	−1,018	320.16	−1,107
318.43	−1,305		
5. pH = 2.40; [Cl⁻] = 0.01 *M*		6. pH = 2.60; [Cl⁻] = 0.01 *M*	
316.47	1,017	318.78	1,119
317.28	816	319.59	920
318.08	543	320.41	680
319.92	370	321.21	466
319.72	90	322.05	135
320.53	−196	322.86	−109
321.30	−487	323.66	−437
322.14	−791	324.46	−769
		325.32	−1,253
7. pH = 3.20; [Cl⁻] = 0.01 *M*		8. pH = 3.60; [Cl⁻] = 0.01 *M*	
328.80	982	331.84	717
329.62	633	332.66	339
330.43	294	333.48	−185
331.22	−3	334.27	−648
332.04	−397	335.09	−1,350
332.86	−909		
333.65	−1,394		
9. pH = 1.6; [Cl⁻] = 0.25 *M*		10. pH = 2.00; [Cl⁻] = 0.05 *M*	
304.38	1,054	306.77	1,725
305.20	919	307.56	1,366
306.00	758	308.38	1,271
306.83	609	309.21	1,082
307.63	474	310.00	864
308.41	289	310.82	742
309.23	114	311.62	499
310.90	−370	312.42	267
311.63	−575	313.25	716
312.43	−819	314.06	−205
313.22	−1,102	314.92	−469
		315.69	−771
		316.52	−1,020
		317.30	−1,762
11. pH = 2.00; [Cl⁻] = 0.03 *M*		12. pH = 3.0; [Cl⁻] = 0.001 *M*	
308.84	1,099	325.03	1,578
309.64	937	325.85	1,420
310.47	710	326.65	1,108
311.26	577	327.47	987
312.08	331	328.27	542
312.89	130	329.08	279
313.73	−146	329.88	82
314.54	−410	330.68	−321
315.35	−653	331.49	−787
316.15	−1,016	332.31	−1,223
316.96	−1,305		

DMSCGN

1. pH = 2.00; [Cl⁻] = 0.01 *M*

Temp, °K	$\Delta F°$, cal/mol	Temp, °K	$\Delta F°$, cal/mol
306.77	−1,547	304.45	−1,045
307.88	−1,830	304.38	−998
306.14	−1,362	304.13	−940
305.59	−1,320	303.80	−890
305.12	−1,164	303.46	−820
304.76	−1,064	303.11	−749
302.74	−700	289.55	570
302.46	−635	289.23	608
302.16	−573	288.96	597
301.96	−513	288.76	629
301.53	−450	288.43	611
301.26	−410	288.04	645
300.97	−367	287.56	653
300.83	−350	287.29	634
300.64	−311	286.96	622
300.38	−273	286.15	650
300.16	−248	285.76	673
299.96	−223	285.01	694
299.73	−173	284.56	682
299.29	−135	284.02	692
298.98	−102	283.83	694
298.76	−65	283.56	674
298.46	−36	283.16	671
297.97	28	282.74	677
297.72	63	282.31	682
297.41	99	281.96	680
297.02	154	281.59	668
296.62	176	280.42	657
296.36	200	280.16	641
296.18	223	279.99	645
295.84	244	279.76	665
295.47	288	279.66	629
295.16	315	279.28	627
294.74	326	278.86	615
294.32	363	278.56	627
293.96	381	278.12	596
293.59	405	277.62	556
293.20	432	277.46	565
292.86	448	277.16	547
292.46	464	277.04	568
292.16	475	276.86	546
291.85	480	276.60	528
291.51	497	276.00	505
291.16	512	275.86	526
290.86	533	275.36	498
290.73	519	274.96	473
290.38	551	274.70	489
289.96	573	274.36	474
		274.16	454

Temp, °K	$\Delta F°$, cal/mol	Temp, °K	$\Delta F°$, cal/mol
2. pH = 2.32; [Cl⁻] = 0.01 *M*		3. pH = 2.60; [Cl⁻] = 0.01 *M*	
313.09	−1,150	319.58	−1,869
311.24	−1,196	315.86	−1,340
309.65	−731	314.75	−1,009
307.47	−274	313.96	−795
305.92	56	311.89	−297
302.63	569	311.11	−144
300.54	788	310.13	70
299.93	1,031	309.04	235
4. pH = 3.00; [Cl⁻] = 0.01 *M*		308.16	362
323.33	−1,859	307.32	512
322.19	−1,463	306.21	641
321.61	−1,309	305.04	805
321.03	−1,139	5. pH = 3.55; [Cl⁻] = 0.01 *M*	
320.54	−929	327.78	−1,467
319.88	−739	326.10	−971
319.26	−558	325.36	−713
318.72	−431	324.39	−449
318.16	−285	323.54	−192
317.62	−172	322.65	42
316.98	−50	321.96	173
316.47	57	320.10	600
315.94	144	320.95	425
314.86	359	319.23	790
314.20	480	318.51	934
313.56	566	317.52	1,131
313.02	652	316.50	1,320
312.50	749		
311.89	842		
311.36	898		
310.75	970		
310.28	1,034		
309.60	1,161		

Table VI (*Continued*)

Temp, °K	$\Delta F°$, cal/mol
6. pH = 3.65; $[Cl^-]$ = 0.01 *M*	
325.37	−554
323.82	−149
322.55	147
321.82	321
320.48	591
319.21	822
318.11	1,157
7. pH = 3.08; $[Cl^-]$ = 0.01 *M*	
321.80	−894
320.59	−501
318.55	−73
316.91	218
315.33	550
313.36	854
311.51	1,129
8. pH = 2.78; $[Cl^-]$ = 0.01 *M*	
317.64	−1,112
315.88	−621
314.38	−250
312.35	155
310.59	474
308.53	788
306.68	1,031
9. pH = 3.10; $[Cl^-]$ = 0.01 *M*	
313.22	1,028
314.02	909
314.84	741
315.64	629
316.47	453
317.27	322
318.08	50
318.88	−102
319.70	−299
320.50	−510
322.13	−940
322.95	−1,115
10. pH = 2.20; $[Cl^-]$ = 0.01 *M*	
298.18	463
298.76	365
299.83	329
300.59	283
301.40	211
302.21	117
303.02	27
303.87	−69
304.72	−201
305.48	−301
306.29	−423
307.11	−585
307.90	−695
308.69	−925
309.49	−1,165
310.34	−1,501
311.10	−1,522
11. pH = 2.31; $[Cl^-]$ = 0.01 *M*	
298.38	719
299.20	636
299.99	576
300.80	514
301.61	419
302.42	387
303.23	302
304.02	198
304.85	61
305.65	−25
306.45	−214
307.28	−353
308.08	−565
308.87	−623
12. pH = 2.59; $[Cl^-]$ = 0.005 *M*	
307.12	1,107
307.94	1,004
308.75	937
309.55	824
310.37	669
311.19	514
311.98	362
312.78	242
313.61	−19
314.43	−173
315.22	−343
316.02	−564
316.83	−713
317.63	−928
13. pH = 2.59; $[Cl^-]$ = 0.003 *M*	
307.97	1,236
310.39	840
311.20	709
312.02	616
312.82	472
314.46	88
315.25	−98
316.06	−247
316.88	−480
317.69	−756
318.46	−963
319.29	−1,354
14. pH = 2.65; $[Cl^-]$ = 0.01 *M*	
304.52	1,199
305.15	1,074
305.94	949
306.77	880
307.60	758
308.36	603
309.14	440
309.96	295
310.82	101
311.63	−106
312.41	−303
313.21	−432
314.05	−662
314.87	−939
15. pH = 1.90; $[Cl^-]$ = 0.013 *M*	
306.21	−1,420
304.17	−1,032
303.02	−837
301.03	−513
298.65	−198
296.95	16
293.44	301
287.87	92
283.38	635
279.01	583
275.54	510
DPC-α-CT	
1. pH = 2.0; $[Cl^-]$ = 0.01 *M*	
307.98	−1,226
306.74	−756
305.76	−484
305.03	−239
303.47	218
304.46	−360
305.08	−284
302.78	392
301.68	653
300.56	950
2. pH = 1.6; $[Cl^-]$ = 0.025 *M*	
303.43	−1,305
301.73	−831
301.71	−853
300.24	−465
300.06	−400
299.31	−254
298.55	−571
297.81	93
296.97	67
296.16	385
295.93	369
295.20	472
294.27	574
293.18	654
291.47	757
289.85	776
288.16	776
285.99	753
283.82	693
280.39	632
277.76	514
275.42	544
273.74	518
3. pH = 2.9 (I); $[Cl^-]$ = 0.01 *M*	
318.80	967
318.19	498
318.33	686
319.35	243
320.24	−159
320.94	−498
321.68	−805
322.57	−1,280
323.48	−1,809
4. pH = 2.05; $[Cl^-]$ = 0.01 *M*	
307.89	−761
306.11	−192
304.43	253
302.75	662
301.06	1,068
5. pH = 2.20; $[Cl^-]$ = 0.01 *M*	
311.80	−1,003
310.84	−652
310.11	−377
309.56	−175
308.40	202
308.96	181
306.18	870
305.61	1,060
6. pH = 2.4; $[Cl^-]$ = 0.01 *M*	
316.92	−1,862
315.49	−1,267
314.12	−732
313.34	−428
312.68	−159
312.06	73
311.26	316
309.85	745
308.43	1,273
7. pH = 2.6; $[Cl^-]$ = 0.01 *M*	
317.53	−952
315.85	−236
314.15	340
312.46	1,025
8. pH = 2.5; $[Cl^-]$ = 0.01 *M*	
316.58	−1,064
315.40	−589
314.07	−53
313.66	101
312.65	500
311.84	807
9. pH = 2.15; $[Cl^-]$ = 0.01 *M*	
309.66	−1,015
308.64	−611
307.36	−185
306.06	156
304.63	648
303.62	920
10. pH = 1.50; $[Cl^-]$ = 0.032 *M*	
303.17	−1,401
301.72	−1,061
300.26	−693
298.93	−417
297.75	−150
296.34	113
295.03	357
293.04	662
11. pH = 1.30; $[Cl^-]$ = 0.05 *M*	
300.15	−1,593
298.06	−1,015
296.75	−717
295.89	−487
294.51	−251
292.14	−23
289.99	244
287.55	374
284.85	343
281.86	195
277.98	252
275.61	−128
274.34	−201
12. pH = 2.90; $[Cl^-]$ = 0.01 *M*	
323.55	1,116
324.19	743
325.04	280
325.37	126
325.76	−86
326.23	−380
326.62	−530
327.24	−921
327.79	−1,193
13. pH = 3.0; $[Cl^-]$ = 0.01 *M*	
321.08	637
320.62	852
321.82	276
322.63	−115
323.30	−420
323.75	−648
324.38	−923
325.17	−1,348
326.12	−1,858
14. pH = 2.90; $[Cl^-]$ = 0.01 *M*	
322.54	−1,270
321.61	−748
320.33	−214
319.84	4
318.77	479
317.86	864
317.15	1,081

Table VI *(Continued)*

Temp, °K	$\Delta F°$, cal/mol	Temp, °K	$\Delta F°$, cal/mol
RNase			
1. pH = 2.00; [Cl⁻] = 0.01 *M*		2. pH = 2.20; [Cl⁻] = 0.01 *M*	
291.90	1,517	297.65	1,343
292.42	1,391	298.48	1,211
293.16	1,279	299.32	1,017
293.93	1,150	300.18	815
294.45	1,077	301.04	650
295.15	918	301.93	452
295.98	758	302.72	297
296.75	633	302.60	90
298.20	346	304.45	−94
299.00	199	305.32	−289
299.74	53	306.19	−502
300.43	−134	307.02	−724
301.21	−301	307.97	−942
300.87	−206	297.05	1,360
300.14	−66	298.08	1,237
299.42	87	299.09	1,026
298.59	247	300.11	817
297.83	376	301.26	595
297.08	535	302.41	370
296.35	765	303.43	146
297.17	535	304.41	−56
299.22	133	305.43	−277
300.25	−66	306.47	−512
301.24	−285	307.48	−727
301.85	−403	308.50	−983
302.64	−580	309.53	−1,266
303.43	−770	310.56	−1,512
304.23	−959	311.56	−1,791
304.94	−1,159		
304.51	−1,024	4. pH = 2.60; [Cl⁻] = 0.01 *M*	
303.84	−869	307.57	959
302.94	−667	308.42	699
301.47	−344	309.28	511
302.30	−519	310.12	301
297.53	442	311.01	92
299.21	132	306.45	1,165
300.89	−217	308.03	789
296.54	632	309.17	522
297.27	466	310.61	173
298.21	326	311.94	−144
298.75	224	312.81	−442
299.78	15	310.87	115
300.61	−154	312.32	−242
302.01	−448	313.05	−431
303.20	−744	314.24	−717
304.51	−1,051	315.34	−1,084

Temp, °K	$\Delta F°$, cal/mol	Temp, °K	$\Delta F°$, cal/mol
316.45	−1,376	5. pH = 2.80; [Cl⁻] = 0.01 *M*	
306.06	1,242	309.97	1,191
309.76	424	310.47	1,050
311.75	−27	310.97	905
313.82	−541	311.44	796
314.74	−825	312.07	617
316.03	−1,109	312.41	536
316.54	−1,233	312.90	402
317.71	−1,586	313.42	289
		313.94	156
3. pH = 2.40; [Cl⁻] = 0.01 *M*		314.43	24
301.64	1,407	315.00	−117
302.75	1,194	315.48	−215
301.94	1,363	316.48	−462
303.14	1,106	316.91	−618
304.27	831	317.47	−740
305.16	647	316.01	−341
306.14	436	318.96	−857
307.16	208	318.46	−1,018
305.20	−24		
309.10	−243		
310.13	−494		
311.11	−710		
303.76	943		
304.67	749		
305.78	508		
306.54	332		
307.64	107		
308.64	−150		
309.67	−382		
310.68	−628		
312.13	−1,017		
312.67	−1,186		
311.67	−857		
313.12	−1,317		
313.56	−1,419		
314.05	−1,498		

without pH correction and 94 kcal/mol with this correction. For CGN in addition to the data already discussed the unfolding kinetics studies of this transition measured by Pohl[21] have also shown this to be the case for CGN.

Validity of the Two-State Approximation. Thus far the experimental complications in establishing the applicability of the two-state approximation appear to be due to the overlay of several types of smaller transitions, known as substate transitions, rather than to the appearance of intermediate states in major unfolding processes. It begins to appear probable that the high degree of cooperativity required for two-state unfolding is a general characteristic of the three-dimensional bonding network of globular proteins. However, it must be noted in this connection that the cooperative unit of the unfolding transition can be smaller than the protein as a whole, *e.g.*, many members of the chymotrypsinogen family have about one-third intact folding in state B. Thus, with larger proteins in addition to problems associated with unfolding before subunit separation or after, we can expect often to find more than one two-state transition taking place under the same experimental conditions. Just as the two-state approximation is a quantitative matter of degree so also the term "all or none" can be deceptive if its use suggests that the total protein forms the cooperative unit for a two-state unfolding process.

Importance of the Heat Capacity. Brandts attributed the heat capacity change on unfolding reactions to the exposure of nonpolar groups to water.[3] This idea can be extended so as to use the heat-capacity change as a measure of the amount of unfolding, presumably the amount of solvated random coil produced in the process. Comparisons of the amounts of fast-moving polypeptide measured by nmr high-resolution line widths and heat capacity values show excellent correlation.[32] The methods show, for example, that several of the chymotrypsinogen proteins are not fully

(32) R. Lumry in "A Treatise on Electron and Coupled Energy Transfer in Biological Systems," T. King and K. Klingenberg, Ed., Marcel Dekker, New York, N. Y., in press.

folded in their best folded state. The dry protein or a protein crystal so incompletely hydrated that there is no interstitial liquid–water phase provides the reference state. Fully hydrated crystals or the protein in solution will have a larger heat capacity insofar as the protein is unfolded. Thus CGN shown by nmr studies to be about 20% unfolded in state A[33] has a heat capacity in solution about 30% higher than the anhydrous or 10% wet crystals studied by Hutchens, Cole, and Stout.[34] α-Chymotrypsin, on the other hand, is fully folded in state A by the nmr measurement and has the same heat capacity in the dry solid as in solution. This use of the heat capacity as a measure of unfolding is very useful. Its application to the kinetics data on unfolding and refolding have shown that the unfolding of chymotrypsin in transition I occurs after the formation of the activated complex.[5,35]

It is possible to carry out van't Hoff studies of higher precision than was obtained in this work. However, baseline uncertainties make it nearly certain that the best of such data cannot be used to determine the form of the relationship between heat capacity and temperature. It appears that we must depend on heat-capacity calorimetry[16] for this relationship. Our raw data are included in Table VI so that more accurate heat capacity and enthalpy values can be computed when the heat-capacity expression is established. Nevertheless the data now available suggest that the enthalpy changes obtained by our method are reliable and that the average $\Delta C_p°$ values we can calculate on the assumption that this quantity is independent of temperature are sufficiently accurate estimates of the average heat capacity change to be of considerable use in estimating the amount of random coil in state A and in state B. Such applications of the data reported in this paper will be made in a subsequent paper of this series.

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Studies of Heme Proteins. II. Preparation and Thermodynamic Properties of Sperm Whale Myoglobin[1]

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Abstract: Preparations of sperm whale myoglobin have been found to contain a tightly bound contaminant which yields abnormally large negative values for enthalpy and entropy of O_2 and CO binding. Characteristics of the contaminant are described. A simple procedure for removing the contaminant is reported. The standard enthalpy changes for O_2 and CO obtained with purified myoglobin are -18.1 ± 0.4 kcal/mol and -21.4 ± 0.3 kcal/mol, while the standard entropy changes were -60 ± 1 and -65.7 ± 0.8 eu/mol, respectively, with a 1 Torr standard state. The van't Hoff enthalpy value for oxygen binding has been confirmed by calorimetric measurements. The results are in good agreement with those of Theorell but not with more recent data, and suggest that some studies of myoglobin have been complicated by contamination.

The use of myoglobin for quantitative studies of oxygen binding, carbon monoxide binding, and linkage mechanisms has been restricted by inconsistencies in the values of the enthalpy and entropy of ligand binding reported by Theorell[2] and by Rossi-Fanelli and Antonini,[3] and by the nonlinearity of van't Hoff plots. Both situations suggest that myoglobin preparations may still be unreliable. The best preparative procedure has appeared to be that of Yamazaki, *et al.*,[4] in which myoglobin is prepared directly in the Fe^{II} form.

Having developed a spectrophotometric method for determining the ligand-binding isotherm at the required level of precision,[5] a study of the nonlinear van't Hoff plots for myoglobin led us to the discovery of an as yet unknown substance or substances which as a result of their interaction with the protein alter the ligand-binding equilibria.[6,7] In this paper we dis-

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