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A Calorimetric Study of the Chymotrypsinogen Family of Proteins*

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ABSTRACT: Several types of calorimetric measurements have been performed on three members of the chymotrypsinogen family of proteins. The heat of protonation of the carboxyl groups of α-chymotrypsin in its best folded state indicates no apparent abnormality in the titration behavior. Such is not the case for chymotrypsinogen A for which an unusually large heat change (~30 kcal/mole) is observed at low pH. This effect is not related to thermal unfolding but is highly indicative of one or more buried carboxyl groups in this protein. The enthalpy changes for the reversible thermal unfolding of chymotrypsinogen at 50° and α-chymotrypsin and dimethionine sulfoxide chymotrypsin at 25 and 40° have been estimated. These estimated values are in good agreement with those obtained from van't Hoff analysis of equilibrium data. This agreement supports the validity of the two-state approximation for the transition. Apparent molar heat capacities for the above three protein species have been determined in their folded and unfolded states. A large increase in heat capacity upon unfolding is observed. Differences in heat capacity between the species in their best folded states are most apparent in the temperature dependence of the heat capacity which are probably related to conformational differences. In the unfolded state the heat capacity was found to be identical for all three species. This latter observation is strong evidence that the unfolded state is thermodynamically similar for all three species.

Present understanding of thermodynamic aspects of protein conformation and conformational changes has been derived principally from van't Hoff studies of reversible thermally induced unfolding at acid pH. Several conclusions which are consistent with the data can be drawn. In some cases such as lysozyme (O'Reilly and Karasz, 1970), ribonuclease (Tsong *et al.*, 1970), and chymotrypsinogen (Jackson and Brandts, 1970) calorimetric data are also available and are in basic agreement with these conclusions listed below. (1) Many globular proteins undergo reversible thermal unfolding *via* a two-state transition.[1] That is, the temperature-dependent equilibrium is an equilibrium between two and only two conformational states; under experimental conditions states of intermediate folding do not exist in any appreciable concentration (Lumry *et al.*, 1966). (2) Unfolding reactions of proteins are accompanied by enthalpy and entropy changes which are extremely temperature dependent because of a large heat capacity difference between the two forms of the protein. This

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[1] The use of the term two state is used to designate an equilibrium between two macroscopic distributions. These two states are not necessarily two well defined structures but can include structural variations within molecules of a given state. Furthermore, the existence of intermediate states is not completely ruled out, but only that the population of such intermediate states is negligible. Details of the meaning of this approximation are thoroughly discussed by Lumry *et al.* (1966) and Jackson and Brandts (1970).

difference in heat capacity is manifested as a nonlinearity of the van't Hoff plot and has been attributed to the exposure of nonpolar residues to the aqueous solvent during the unfolding process (Lumry and Biltonen, 1969; Brandts, 1969; Tanford, 1968). (3) From previous studies of the thermal unfolding of various chymotrypsinogen proteins it has been found that the change in heat capacity varies from protein to protein. These differences in ΔC_p° have been interpreted as due to variation in the size of the cooperative unfolding unit of the protein (Biltonen and Lumry, 1965, 1969, 1971). (4) It has also been concluded that the thermally unfolded state, state B, of several members of the chymotrypsinogen family of proteins is experimentally indistinguishable (Biltonen and Lumry, 1971). Thus the number of nonpolar residues unfolded in state B of these proteins is presumably the same, and hence the heat capacity in the unfolded state should be identical for all. This suggests that the variation in the thermodynamic parameters for unfolding among several proteins of the chymotrypsinogen family is the result of differences in the conformation of the proteins in their best folded states, state A.

It is obvious that studies of thermodynamic changes during protein transconformation reactions can potentially yield useful information regarding the nature of the conformational change. Such changes in thermodynamic functions have generally been obtained by van't Hoff analysis of equilibrium data. However, any thermodynamic analysis of equilibrium data requires a number of assumptions regarding the transition, particularly that the process must be of the two-state type. For example, if the transition is not of the two-state type then the calculated thermodynamic changes for the transition have no direct physical interpretation. It has also become apparent that an important thermodynamic quantity is the heat capacity change during these transconformational reactions, and by necessity these must usually be determined from the second derivative of the actual experimental data. If the equilibrium data are not of sufficiently high precision to obtain reliable second derivatives, then heat capacity changes cannot be reliably estimated. For these reasons it is important to supplement van't Hoff studies of conformational transitions by direct calorimetric measurements of the enthalpy and heat capacity changes as has been done for chymotrypsinogen, ribonuclease, and lysozyme. Clearly, a comparison of equilibrium and calorimetric values for enthalpy and heat capacity changes can provide, first, a test of the two-state hypothesis and second, additional information about the nature of the states involved and the process of conformational change. In this communication calorimetric studies of three members of the chymotrypsinogen family of proteins will be reported. The results are found to provide strong support for the various conclusions derived from equilibrium studies. In addition these studies demonstrate that presently available calorimetric equipment can be used for extensive thermodynamic studies of proteins with reasonable amounts of material.

Experimental Section

Chymotrypsinogen (CGN)[2] and α-chymotrypsin (CT) were obtained from Worthington Biochemical Corp., Freehold, N. J. Dimethionine sulfoxide chymotrypsin (DMS-CT) was prepared from α-chymotrypsin according to the method of Schachter and Dixon (1964). Chromatography according to the method of Hirs (1955) indicated all preparations to be better than 90% homogeneous. α-Chymotrypsin exhibited 90% and the dimethionine sulfoxide derivative 70% all-or-none enzymatic activity according to the cinnamoylimidazole assay (Schonbaum *et al.*, 1961). All preparations were used without further purification. The importance of impurities in the preparations will be discussed later.

Protein solutions (2.0–10 mg/ml) were prepared in a 0.01 M solution of KCl in distilled, deionized water. The pH was adjusted to the desired value with 0.1 M HCl or NaOH. The protein concentration was determined by optical density measurements at 282 nm assuming an extinction coefficient of 19.5 l. (g cm).

Enthalpy Measurements. Measurements of the molar enthalpy change were carried out with two different microcalorimeters based on the heat conduction principle. Enthalpy measurements were made with a batch microcalorimeter (Wadsö, 1968) similar to the LKB Model 10700-2 and a Model 10700-1 flow microcalorimeter (Monk and Wadsö, 1968) kindly made available by LKB Instruments, Inc., Rockville, Md. For the runs made with the batch microcalorimeter, a portion of protein solution (usually about 2.5 g) was weighed into one side of the sample cell, by difference, from a hypodermic syringe. Sufficient HCl solution (usually about 5.0 g) of appropriate concentration (0.020–0.034 M) to bring the final pH to near 2.00 was similarly weighed into the other side. The reference or blank cell contained roughly the same total mass of water. After attainment of thermal equilibrium the calorimeter assembly was rotated in a reproducible manner to effect the mixing of the two solutions. When the post-run equilibrium had been achieved, several additional rotations were performed to check for completeness of reaction and to permit calculation of the differential frictional heat of mixing. Following the run, the solution was removed from the cell and its pH redetermined at the appropriate temperature. In practice, final pH values ranged between extremes of 1.92 and 2.08, with the majority of solutions being within 0.02 pH unit of 2.00. In typical experiments heat quantities of 10–100 mcal were involved.

In experiments on the flow calorimeter protein and acid solutions were pumped through the cell at equal rates of about 0.003 ml/sec which were specifically checked for each experiment. The pH of the effluent stream was measured to determine the final pH of the reaction mixture. The heat of the reaction was manifested as a steady-state emf. This emf when compared to the steady-state emf generated by only water flowing through the cell provided a measure of the heat or reaction. Generally, heat fluxes of 10–100 μcal/sec were observed.

Both calorimeters were calibrated electrically. The accuracy of the electrical calibration and the performance of the calorimeters were checked by measuring the heats of dilution of urea and sucrose. In all experiments the heats of dilution of acid and protein solution were determined independently and subtracted from the heat of mixing when significant.

Heat Capacity Measurements. Calculations of apparent heat capacities for CGN, CT, and DMS-CT in states A and B were based upon calorimetric measurements of specific heats at pH 2.00 and 4.00 and the appropriate temperature. These experiments were carried out on solutions containing approximately 1 g of protein in 100 ml of 0.01 M KCl, using an LKB 8700 precision calorimetry system (Sunner and Wadsö, 1966). The procedure used with each solution was as follows. First the protein solution, at approximately 25°, was introduced into the calorimeter vessel from a weight buret. The calorimeter was then assembled and placed into a bath

[2] Abbreviations used are: CGN, chymotrypsinogen; CT, α-chymotrypsin; DMS-CT, dimethionine sulfoxide chymotrypsin.

thermostatted at 25.00 ± 0.01°. Either cold, dry air passed over the vessel or the calorimeter heater was used to bring the temperature of the solution to the starting temperature of the experiment, approximately 24.9°. During the fore-heating period of about 7 min measurements of resistance *vs.* time were recorded. At a predetermined value of the resistance (and, hence, the temperature) the heater was automatically turned on. It remained on for exactly 5 min and then was automatically shut off. It should be noted that all heating periods for all specific heat determinations and calibrations at 25° commenced at exactly the same temperature and involved the same electrical heat input. Thus, the final temperature was the only variable among the measured quantities. This final temperature was determined from reading of resistance as a function of time in the after-heating period. After the completion of the above sequence of steps, the calorimeter vessel and its contents were cooled with a stream of cold air until the temperature was again approximately 0.1° below that of the bath. The complete calorimetric run was then repeated. From three to five such runs were initially made on a single solution at 25°.

After the reproducibility of repeated determinations was established as within one part in 10^4, the calorimeter jacket was removed from the bath and opened, and the vessel was warmed to the desired temperature with a forced hot air heater and the internal heater. This process required less than 5 min, and thus should not have contributed significantly to aggregation or autolysis. Once temperature had been achieved, the vessel was returned to the jacket, which was now placed in another bath thermostatted at the higher temperature ±0.01° (40.00 ± 0.01° for CT and DMS-CT; 50.00 ± 0.01° for CGN). Again a series of three to five heat capacity measurements was carried out, employing the previously described techniques. When these were completed, one or two additional runs at 25° were performed on the same filling of the calorimetric vessel in order to check reproducibility before and after exposure to high temperature.

In the course of this work four such complete series were performed for each protein, two on separately prepared solutions of pH 2.00 and two on separate solutions of pH 4.00. Calibration runs with distilled, deionized water were carried out in exactly the same manner.

Results

Enthalpy Measurements. If a protein solution is mixed with sufficient HCl to reduce the pH to some specific value, heat will be evolved or absorbed. This heat includes contributions from the heat of dilution of the various components of the system which can be measured independently and subtracted from the observed heat changes. The remaining heat of reaction, ΔH_a, is composed of two parts, that due to titration of the protein in its original conformational state, ΔH_t, and any heat change produced by a conformational change of the protein, ΔH_c. These heat changes to be reported below have been referred to pH 4.0 as a reference state. pH 4.0 has been selected as the reference state because at this pH all three protein species are found in their best folded state, state A,[3] under all conditions of temperature used in this study. The salt concentration of 0.01M KCl was chosen for these studies because previous work had shown that these proteins exist as primarily monomeric species at this salt concentration at pH values less than or equal to 4.0 (Biltonen, 1965).

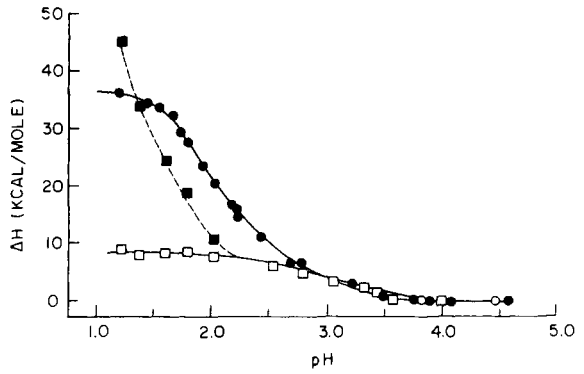
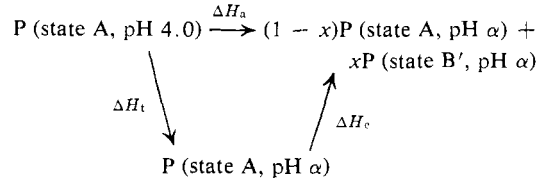


FIGURE 1: Enthalpy titration curves of chymotrypsinogen A (●) and α-chymotrypsin (■). Results for α-chymotrypsin corrected for partial unfolding at low pH (□). See text for details.

HEAT OF TITRATION AT 25°. The overall protein (P) reaction can be written as



where x is the fraction of protein molecules undergoing a conformational change to some new state B′ at pH α, the final pH. In principle, ΔH_t can be directly determined if the mixing reaction is carried out under conditions where no conformational changes occur, *i.e.*, $x = 0$ and $\Delta H_a = \Delta H_t$. Then, if the mixing reaction is performed under conditions such that $x = 1$, ΔH_c can be calculated without any assumptions regarding the nature of the conformational change.

Values of ΔH_a for CT as a function of pH at 25° are summarized in Figure 1. Each ΔH_a is the total heat required to titrate the protein from pH 4.0 to the indicated pH. At this temperature CT is completely in state A at pH 4.0, but at lower pH it exists as an equilibrium mixture of state A and state B, the thermally unfolded state. Thus ΔH_a, at the lower pH values, is equal to $\Delta H_t + x\Delta H_c$, where x = the fraction of the molecules in state B and ΔH_c is the enthalpy for transition I at this temperature. These experiments provide a series of heat measurements described by the set of equations: $\Delta H_a = \Delta H_t + x_i\Delta H_c$, where x_i = fraction protein unfolded at a particular final pH value. If we assume ΔH_t is identical for two pH values, a reasonable assumption at pH ≤2.0, and since x_i has previously been determined using ultraviolet spectral data (Biltonen and Lumry, 1969) each pair of observations represented by a pair of equations as given above can be solved for ΔH_c and ΔH_t. Doing so for our values of ΔH_a below pH 2 we obtain estimates of $\Delta H_c = 50 \pm 10$ kcal/mole

[3] State A is the low-temperature form of the protein and also has been referred to as the best-folded state or "native" state. The primary criteria for judging the purity of this state, relative to the presence of any significant amount of state B or denatured form, have been the ultraviolet absorption pattern and solubility in high salt buffer. The solubility property (Eisenberg and Schwert, 1951) is the most definitive since in state A the protein is completely soluble whereas in state B it is essentially insoluble. A more complete experimental description is given by Brandts (1964) for chymotrypsinogen and Biltonen and Lumry (1969, 1971) for chymotrypsin and dimethionine sulfoxide chymotrypsin.

TABLE I: Enthalpy of Unfolding (kcal/mole).

Protein	Temp (°C)	ΔH_a[d]	ΔH_c	$\Delta H_c'$	ΔH (van't Hoff)
	25		50 ± 10		50
α-Chymotrypsin	40	119 ± 6[e]	110	105[g]	120[a]
Chymotrypsinogen	50	160 ± 3[e]	123	116[g]	122[b]
Dimethionine sulfoxide chymotrypsin	25	41 ± 3[e]	32		36[c]
	40	82 ± 2[f]	73	68[g]	72[c]

[a] From Biltonen and Lumry (1969). [b] From Brandts (1964). [c] From Biltonen and Lumry (1971). [d] These values represent averages obtained from several preparations. [e] Averages of results obtained using both the batch and flow calorimeters. [f] The results represent only results obtained with the flow calorimeter. At this temperature measurements with DMS-CT on the batch calorimeter provided consistently low values. This is most likely attributable to irreversible aggregation of the protein occuring during the 1.5 hr premixing equilibration period. Additional evidence for this conclusion is provided by the fact that with this calorimeter, ΔH_a was found to decrease with increasing dimethionine sulfoxide chymotrypsin concentration. We thus conclude that for this protein at 40° the enthalpy value obtained with the flow calorimeter is the more reliable. It should further be noted that no significant dependence of enthalpy upon protein concentration was observed for dimethionine sulfoxide chymotrypsin in the flow calorimeter or for the other proteins in either calorimeter. [g] These values have been calculated assuming ΔC_p° for protonation of carboxyl groups on the proteins is similar to that of acetic acid.

and $\Delta H_t = 9 \pm 1$ kcal/mole. The former value is in agreement with the van't Hoff estimate of ΔH_c. Using $\Delta H_c = 50$ kcal/mole and known values of x, the values of ΔH_a have been corrected to provide a representation of ΔH_t as a function of pH as shown in Figure 1. It is thus found that at pH values less than 2.2 $\Delta H_t = 9 \pm 1$ kcal/mole, which represents ΔH_t (pH 4 → complete protonation) in state A.

Values of ΔH_a for CGN as a function of pH at 25° are also summarized in Figure 1. At this temperature CGN is completely in state A[3] at all pH values above pH 1.5. Hence $\Delta H_a = \Delta H_t$ over the pH range 4.0–1.5. The single value of ΔH_a at pH 1.2 in Figure 1 has been corrected to account for partial unfolding. ΔH_t (pH 4 → complete protonation) for CGN at 25° is thus estimated to be 37 kcal/mole. This result is in agreement with recently reported results of Shaio and Sturtevant (1970).

HEAT OF UNFOLDING. ΔH_c for the thermal transition of CT at 25° has been estimated to be 50 kcal/mole as previously discussed. The error in this estimate is quite large because complete unfolding could not be effected at this temperature except by extremely low pH where some irreversible denaturation is known to occur.

At 40° CT exists completely in state A at pH 4.0 and completely in state B at pH 2.0. Thus ΔH_a associated with titration of CT from pH 4.0 to 2.0 at this temperature is the sum of $\Delta H_t + \Delta H_c$. If it is assumed that ΔH_t is temperature independent, ΔH_c can be calculated at 40° for CT using the calorimetrically obtained values of ΔH_a and $\Delta H_t = 9$ kcal/mole. However, this assumption may be incorrect insofar as it is known that the heat of protonation for small carboxylic acids is quite temperature dependent. ΔC_p° for protonation of acetic acid has been found to average approximately 30 cal/(mole deg) (Leung and Grunwald, 1970) over the temperature range 25–50°. Using this estimate for the ΔC_p° of protonation of ten carboxyl groups of CT which protonate between pH 4 and 2, ΔH_t at 40° is estimated to be 14 kcal/mole.

The experimental values of ΔH_a and derived values of ΔH_c for CT assuming ΔH_t is temperature independent and assuming ΔC_p° per carboxyl group is the same as that of acetic acid are summarized in Table I along with van't Hoff estimates of ΔH_c for transition I.

DMS-CT, produced by oxidation of methionines-180 and -192 of CT, is found in state B at 25° or higher at pH 2.0, although it exists in its best folded state at pH 4.0 at both 25 and 40°. ΔH_a values for DMS-CT at 25 and 40° are summarized in Table I. Although ΔH_t (pH 4–2) for DMS-CT cannot be determined at these temperatures, ΔH_c can be calculated if it is assumed that ΔH_t is identical for DMS-CT and CT. The experimental results and calculated values of ΔH_c for transition I of DMS-CT are tabulated in Table I along with the van't Hoff estimates.

At 50° CGN exists completely in state A at pH 4.0 and completely in state B at pH 2. Thus ΔH_a at this temperature is the sum of $\Delta H_t + \Delta H_c$. ΔH_c has been calculated at 50° assuming ΔH_t is temperature independent and also assuming ΔC_p for ten carboxyl groups is 300 cal/(mole deg) and compared to the van't Hoff estimates. These results are also tabulated in Table I.

Heat Capacity Measurements. In calculating the apparent molar heat capacity of the protein, the bulk solvent, 0.01 M KCl at pH 2.00 or 4.00, was assumed to have its experimentally determined specific heat. In the case of the pH 2.00 protein solutions, a significant quantity of Cl^- counterions was introduced with the 0.1 M HCl used to adjust the pH, and it thus became necessary to make a correction for the contribution of these ions to the observed heat capacity of the final solution. For example, a solution prepared from 1 g of protein and an initial weight of 100 g of the 0.01 M KCl–0.01 M HCl solvent solution required about 10 g of 0.1 M HCl to restore the pH to 2.00. The titrating protons were considered to be part of the protein and since the pH adjustment kept the hydrogen ion concentration in the bulk solution at a constant value, no correction was made for these added ions. On the other hand, the added chloride ions associated with the protein as counterions were assumed to contribute to the total heat capacity of the system. Calculation of this contribution was based upon the added quantity of these excess ions and tabulated values for the apparent molar heat capacity of Cl (National Bureau of Standards, 1965). Thus, for 10 g of 0.1 M HCl, the heat capacity contribution due to the Cl^- counterions was approximately −0.04 cal/deg. This is a significant amount, since the difference in heat capacity between solvent and protein solution is only about 0.5 cal/deg. The contribution of the titrating protons to the mass of the protein was negligible. It was, however, necessary to make a small correction for the mass of water introduced with the protein.

The experimentally determined heat capacity of the calorimetric system, C, can thus be written as the sum of individual contributions from the calorimeter, the solvent (0.01 M KCl 0.01 M HCl), the counterions, and the protein: $C = C$(calorimeter) + C(solvent) + $C(Cl^-)$ + C(protein). C(calorimeter) is experimentally determined from calibrations on pure water.

TABLE II: Apparent Molar Heat Capacities of Chymotrypsinogen Proteins (kcal/(mole deg)).

Protein	State A (pH 4)			State B (pH 2)		
	25°	40°	50°	25°	40°	50°
α-Chymotrypsin	9.6 ± 0.3	10.2 ± 0.5			13.5 ± 0.2	
Chymotrypsinogen	9.4 ± 0.3		10.6 ± 0.5			13.6 ± 0.3
Dimethione sulfoxide	9.4 ± 0.2	11.5 ± 0.3		13.7 ± 0.1	13.4 ± 0.3	

C(solvent) is calculated from the known mass of the solvent (including the HCl solution added in pH adjustment) and its measured specific heat of the solvent, and $C(Cl^-)$ is calculated as previously indicated. The above equation can then be solved for the C(protein). From this value, the apparent specific heat and the apparent molar heat capacity of the protein can be obtained. For the latter calculation, a molecular weight of 24,600 g was assumed. Values of apparent molar heat capacity for the different protein species under a variety of conditions are tabulated in Table II.

Discussion

Sources of Error. The random errors in the various calorimetric experiments described herein are indicated in error estimates tabulated in Tables I and II and in the text. The magnitudes indicated are within acceptable limits considering the precision of the calorimetric measurements and the reproducibility of preparing the protein solutions. However, in the comparison of the calorimetric estimates of thermodynamic quantities with estimates derived by van't Hoff analysis systemic errors are of the most significance. The more important of these latter sources of error are discussed individually below.

In the calculation of all quantities ultraviolet absorption has been used to determine protein concentration. Thus any error in the assumed value of the extinction coefficient would produce a systematic error in the calculated value. The magnitude of error estimated in the extinction coefficient is, however, on the order of 2% and does not seriously affect our results.

A more important source of systematic error is the presence of protein impurity. Generally such impurities may be regarded as inert with regard to the structural transitions (*e.g.*, the pH transition of CGN at 25° and thermal unfolding). If this is the case then the amount of protein undergoing the transition is less than the total protein and our estimates would consequently be lower than the true value. This problem could seriously affect the validity of our conclusion that transition I is a two-state transition for these protein species. However, for CT and CGN this does not appear to be serious since characterization of these proteins indicates a better than 90% purity. This is not the case for DMS-CT and for this reason we have only tentatively concluded that the two-state approximation is valid. This point is discussed in more detail later. Impurities will most likely have a smaller relative effect on the estimates of the apparent heat capacities since the difference between the measured heat capacity and the true heat capacity of the pure protein will be equal to the product of the fraction of impure protein times the difference in heat capacity between pure protein and the impurity.

Association of the proteins is another possible source of systematic error in our values. The extent of such an error appears to be small. First, conditions were carefully selected such that the protein species existed in a monomeric state under all conditions of the experiment. Secondly, no concentration dependence of the reported enthalpy measurements was observed indicating association was negligible as regards heat effects. The exceptions to these situations were attempted measurements on the batch microcalorimeter as discussed in the footnote to Table I. Irreversible aggregation of the proteins, a phenomenon which is quite probable in the unfolded state, could also cause problems. However, this is a kinetically slow process compared to the conformational processes of present interest and would not be of significance in experiments with the flow microcalorimeter. If they were of significance they would cause major problems only in the batch microcalorimetric experiments. With the exception of DMS-CT, we have noted no difference between the two types of experiments and hence concluded that irreversible aggregation is also negligible.

Association of the proteins would probably be of most significance in the heat capacity measures for at least three reasons. (1) Irreversible association would have a significant influence on the precision of the experiments primarily because of abnormal baseline drifts. Upon consideration of the precision of our experiments, however, we conclude this to be insignificant. (2) If reversible association exists an anomalous contribution to the heat capacity will be made due to temperature induced changes in the equilibrium. That is, during the experiment a portion of the heat absorbed will be due to a shift in the equilibrium position of the association reaction. The magnitude of this anomalous contribution will be on the order of $\Delta H^2/RT^2$, where ΔH is the standard enthalpy change for the association reaction, R the universal gas constant, and T the absolute temperature. If $\Delta H \simeq 10$ kcal/mole, a number suggested from calorimetric results for the association of CT at neutral pH (Shaio and Sturtevant, 1969), the anomalous contribution will be on the order of 0.1 kcal/(mole deg). This is well within the present experimental precision and for this reason such contributions are deemed insignificant. (3) If association of, for example, the unfolded protein resulted in removal of hydrophobic groups from interaction with the aqueous environment then our heat capacity estimates would be only lower estimates. The magnitude of such an error would be equal to the fraction of protein aggregated times the difference in heat capacity between the aggregated protein and the monomeric protein. For reasons discussed previously this is not regarded as a serious problem.

Heat of Titration. Addition of excess hydrogen ion to solutions of the chymotrypsinogen proteins at pH 4 will protonate approximately ten carboxylic groups. On the basis of heats of protonation of simple carboxylic acids, the total heat of protonation of these groups on the protein will be small if the protein does not undergo a conformational change or the

groups which protonate are not abnormal with respect to the heat of ionization. For ribonuclease at 25° (Krescheck and Scheraga, 1966) the heat of protonation of carboxylic groups was found to be approximately 6 kcal/mole for 11 such groups under conditions where no conformational change occurred. This value is consistent with normal behavior of the acid groups and likewise the heat of protonation of CT in state A at 25° appears to be normal (ΔH_t = 9 kcal/mole). On the other hand this is not the case for CGN. The results for the latter protein suggest that an unusual (conformational) change, with an apparent pK of approximately 2.5 at 25°, occurs on protonation of some of the carboxylic acid groups of the protein. From the present data it is difficult to ascertain the number of groups involved in this abnormally large heat effect. Either a single group with an apparent heat of protonation of about 30 kcal/mole or several groups, acting independently with accordingly smaller heats of protonation per group, could be involved in the observed effect. Preliminary studies of the heat of protonation of CGN at higher temperature suggest that a large number of independent groups are not responsible for this heat effect since at 36° the apparent pK for this "transition" is shifted by approximately 0.6 pH unit.

This apparent stuctural change of chymotrypsinogen at low pH is not accompanied by any significant change in the ultraviolet absorption spectra or solubility in high salt buffer and hence is completely different from the reversible thermal unfolding monitored by spectroscopic changes (Brandts and Lumry, 1963; Brandts, 1964). However, small changes in the optical rotatory dispersion pattern of the protein below pH 3.2 (Biltonen *et al.*, 1965) have been observed. These changes have not been studied in detail but presumably are related to the same transition. It is also quite likely that the groups involved in triggering the transition include the abnormal carboxyl groups found by Delaage *et al.* (1968) and Carraway *et al.* (1969). Specifically, the unusual heat may involve the protonation of asparatic acid residue 194 which is buried in CGN and hydrogen bonded to histidine-40 according to recent X-ray analysis of the crystal structure (Freer *et al.*, 1970). Protonation of this carboxyl group could indeed involve a conformational rearrangement of the protein molecule. The local interaction of this aspartic acid residue presumably plays an important role in the activation of the zymogen to active enzyme and the differences in the acid titration heats between CT and CGN may be indicative of the conformational differences between the two proteins. Further studies are currently under way to more clearly define this phenomenon.

Transition I. We have found that the enthalpy change for the thermal unfolding of three chymotrypsinogen proteins as determined calorimetrically is in good agreement with the van't Hoff heat for the reaction as determined from equilibrium studies. This agreement provides strong support for the validity of the two state approximation for transition I of all three proteins. The estimates obtained assuming ΔH_t is temperature independent are in slightly better agreement; estimates obtained assuming $\Delta C_p°$ for protonation of the carboxyl groups is similar to that for acetic acid are consistently lower than the van't Hoff estimates. This does not invalidate the conclusion that transition I is a two state transition however since it has been shown that: ΔH(calorimetric) $\geq$ ΔH(van't Hoff). If the reaction is a multistate transition then the inequality holds. If it is true that the ΔH_c calculated assuming ΔH_t is temperature dependent is correct, the slight discrepancy between the calorimetric and van't Hoff heats cannot be solely explained by the existence of a multistate transition, but is better explained by the presence of impurity in the protein preparation of 10% or less. We therefore conclude that the van't Hoff and calorimetric heat for transition I are identical within the present experimental errors, and that the two-state approximation is valid in this case.

In calculating ΔH_c for chymotrypsinogen at 50°, we have assumed that ΔH_t (pH 4–2) = 37 kcal/mole is either temperature independent, or only slightly temperature dependent. This is reasonable since the degree of protonation at pH 4.0 will not change significantly from 25 to 50°, and also both the low temperature and high temperature form will be essentially totally protonated at pH 2, 50°. Thus ΔH_c calculated from our data corresponds to the enthalpy change from state A to state B, under conditions of complete protonation. The fact that this value is in close agreement with the van't Hoff value supports the two-state approximation for transition I. The overall reaction scheme for chymotrypsinogen at acid pH can be written as: A $\rightleftarrows$ A′ $\rightleftarrows$ B. State A is the form found at low temperature (<25°), pH >3.2. State A′ is the completely protonated form of the protein, which is indistinguishable from state A with regard to its solubility characteristic but is of higher enthalpy by approximately 30 kcal/mole. We have found, and are supported by the data of Jackson and Brandts (1970), that the transition A′ $\rightarrow$ B is a two-state transition within the limits of our present experimental errors. The existence of the states A and A′ does not invalidate this conclusion since the equilibrium between states A and A′ will only introduce an apparently larger heat capacity for the low temperature state, but the magnitude of this "between states" heat capacity is still probably beyond present limits of measurement.

The calculated values of ΔH_c for DMS-CT may include significant systematic errors from two possible sources. (1) The enzyme preparation is impure, but we have calculated ΔH_c on the basis of moles of total protein. If a fraction of the protein does not undergo any conformational change our calculated value is a lower estimate. (2) ΔH_t for dimethionine sulfoxide chymotrypsin was assumed to be equal to that for chymotrypsin. If the latter assumption is incorrect another systematic error is introduced into our calculated value of ΔH_c. Because of the potential of these systematic errors we can only tentatively conclude that the thermal transition of DMS-CT is a two-state conformational change. Substantiation of this particular point awaits purification of the protein which is currently under way. However, the temperature dependence of ΔH_c for this species is independent of the above sources of systematic error and we can conclude that the $\Delta C_p°$ for the transition is greater than zero, a point which is substantiated by direct heat-capacity measurement. The fact that $\Delta C_p° > 0$ for thermal unfolding of globular proteins has been deduced previously from van't Hoff analysis of the transition (Brandts, 1969).

Heat Capacities. Few values of absolute heat capacities of proteins exist. The apparent specific heat values of the chymotrypsinogen familty of proteins (0.38 cal/(g deg) at 25°) can be compared with the values of 0.40 at 11° (Bull and Breeze, 1968) and 0.46 at 25° (Krescheck and Benjamin, 1964) obtained for ovalbumin. Our value for CGN is in agreement with that obtained by Jackson and Brandts (1970) (0.40) and can be compared to the value obtained by Hutchens *et al.* (1969) for the anhydrous protein (0.31).

Our values of the heat capacities of all three proteins in states A and B show that upon thermal unfolding there is a concomitant increase in the heat capacity of the protein. This result is in agreement with a similar conclusion deduced from the temperature behavior of the van't Hoff heat for the transi-

tion and is in agreement with calorimetric experiments on the thermal unfolding of CGN (Jackson and Brandts, 1970) and ribonuclease (Tsong *et al.*, 1970). Values for the heat capacity change during this transition, calculated from the results in Table II, can be compared to previously obtained values and good agreement is found. The increase in heat capacity has been attributed to the interaction of exposured nonpolar residues in state B with the aqueous medium.

We have found that at 25° the heat capacity of all three protein species in state A is approximately identical. However, each possesses an apparently strong positive temperature dependence. This result is in agreement with the data of Jackson and Brandts (1970) for CGN. The temperature dependence of the heat capacity for each species is also apparently different, which may indicate that the structure of each protein in state A is different. This would be in agreement with similar conclusions previously deduced from a number of other studies (Biltonen and Lumry, 1971; Hollis *et al.*, 1967).

The heat capacity of each protein in state B, the thermally unfolded state, is identical. This result suggests that the conformations of each species in state B are thermodynamically identical, which is also in accord with the results of previous studies (Biltonen and Lumry, 1971). Although the amount of data is severely limited no temperature dependence of the heat capacity in state B was observed, whereas in state A the temperature dependence of apparent molar heat capacity was found to be positive. Thus the heat capacity change for thermal unfolding will show a negative temperature dependence, a conclusion in agreement with the results of Jackson and Brandts (1970) for the thermal unfolding of CGN. In previous semiempirical analysis of the thermodynamics of the unfolding of globular proteins it has been assumed that either the heat-capacity change for the transition is positively dependent upon temperature (Biltonen and Lumry, 1969, 1971; Brandts, 1964) or temperature independent (Shaio, 1968). Although this aspect is of minor importance with regard to the validity of the analysis, it now appears that both assumptions are incorrect.

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