and methylation to XXb.[7,36,49] The relative and absolute configurations of these compounds have been established.[7,36]

Acknowledgments. We thank Professor H. Edery and Dr. Y. Grunfeld of the Biological Institute, Ness-Ziona, Israel for the psychopharmacological tests, Dr. N. Danieli for the mass spectra, Miss I. Ramati and Mrs. N. Shoef for their excellent technical help, and the Israeli Police for the supply of confiscated hashish. The last stages of the above research were supported by the National Institute of Mental Health (Grant No. MH-13180) whom we thank.

(49) J. G. Smith and G. F. Wright, *J. Org. Chem.*, **17**, 1116 (1952).

Studies of the Chymotrypsinogen A Family of Proteins. VIII. Thermodynamic Analysis of Transition I of the Methionine Sulfoxide Derivatives of α-Chymotrypsin[1]

Rodney Biltonen and Rufus Lumry*

Contribution from the Laboratory for Biophysical Chemistry, Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received August 15, 1969*

Abstract: Spectral changes at 293 nm have been used to monitor the first thermal-unfolding transition (transition I) of the monomethionine sulfoxide and dimethionine sulfoxide derivatives of α-chymotrypsin. From these data $\Delta F°$, $\Delta H°$, and $\Delta S°$ have been calculated as a function of pH and temperature. Monomethionine sulfoxide chymotrypsin and its parent, chymotrypsin, show identical transition I characteristics. On the other hand, dimethionine sulfoxide chymotrypsin is thermodynamically less stable than its parent although transition I still exhibits all-or-none cooperativity. The thermodynamic results are in complete accord with the predictions of Brandts' "force" analysis of protein unfolding and provide strong support for this type of analysis. For example, dimethionine sulfoxide chymotrypsin exhibits a temperature of maximum stability which is a characteristic consequence of a change in the number of interactions between water and the nonpolar moieties of the protein which occurs on unfolding. Parameters of Brandts' analysis of transition I evaluated with the aid of model compound data allow comparisons among α-chymotrypsin, chymotrypsinogen, and dimethionine sulfoxide α-chymotrypsin to be made. The cooperative unfolding units of dimethionine sulfoxide α-chymotrypsin and chymotrypsinogen are approximately one-half that of α-chymotrypsin. The results are consistent with results obtained by other investigators using nuclear magnetic resonance line widths as a measure of segmental flexibility and calorimetric measurements of enthalpy changes and heat capacity. It appears that the thermally unfolded states of all the chymotrypsin proteins thus far studied are very similar, although a significant amount of folded structure is retained in this state. Since the cooperative unfolding unit of dimethionine sulfoxide chymotrypsin is only about half that of its parent, this protein must be partially unfolded in its best folded state. The change in enzymic efficiency of dimethionine sulfoxide chymotrypsin may be related to this partial unfolding which apparently must be restored before chemical catalysis can take place.

Although chemical and quantitative understanding of the unfolding processes of small globular proteins is very incomplete, some important phenomenological details having general significance have been established. Specifically we may list: (1) as first noted by Brandts,[2,3] these transitions are marked by large heat-capacity changes so that the van't Hoff isochores have marked curvature and may in some cases show a maximum in free energy of unfolding. In such cases unfolding can be produced by lowering as well as raising the temperature from the "temperature of maximum stability." (2) Some proteins demonstrate simple "two-state" behavior[4] which means that only two macroscopic states need be considered, the folded state, hereafter called state A, and the unfolded state, state B.[5,6] (3) Unfolding to the completely unfolded state need not occur in a single step. Ribonuclease A[7,8] and α-chymotrypsin[9-11] on increasing temperature experience two-state transitions which produce only a partial unfolding. Complete unfolding of chymotrypsin has not been effected by raising the temperature although it occurs in 8 *M* urea.[12] (4) The dominant characteristic of two-state transitions is the high degree of cooperativity but proteins of the same or nearly the same amino acid sequence, such as chymotrypsinogen A (CGN) and α-chymotrypsin (CT),[10] can have different numbers of

* To whom correspondence should be addressed.

(1) This is publication No. LBC 33. Request reprint by number. The work was supported by the U. S. Public Health Service through National Institutes of Health Grant No. 2 R01 AM 05853 and by fellowship support from the 3M Corporation to R. B. This work is reported in full in the Ph.D. Dissertation of R. Biltonen, University of Minnesota, 1965. A preliminary report of this work has appeared (R. Biltonen and R. Lumry, *J. Amer. Chem. Soc.*, **87**, 4208 (1965)).

(2) J. Brandts, *J. Amer. Chem. Soc.*, **86**, 4291 (1964).

(3) J. Brandts, *ibid.*, **86**, 4302 (1964).

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

(5) R. Biltonen and R. Lumry in "Structure and Stability of Biological Macromolecules," S. Timasheff and G. Fasman, Ed., Marcel Dekker, New York, N. Y., 1969, p 65.

(6) R. Biltonen, R. Lumry, V. Madison, and H. Parker, *Proc. Nat. Acad. Sci. U. S.*, **54**, 1412 (1965).

(7) J. Brandts, *J. Amer. Chem. Soc.*, **87**, 2759 (1965).

(8) J. Brandts and L. Hunt, *ibid.*, **89**, 4826 (1967).

(9) R. Biltonen and R. Lumry, *ibid.*, **91**, 4251 (1969).

(10) R. Biltonen and R. Lumry, *ibid.*, **91**, 4256 (1969).

(11) R. Biltonen, A. T. Schwartz, and I. Wadso, manuscript in preparation.

(12) R. Biltonen, Ph.D. Dissertation, University of Minnesota, 1965.

cooperating residues, that is, cooperative units of different size in unfolding reactions. (5) In the absence of cosolvents and aggregation in state B the cooperative transitions are the same whether produced by changes in pH, solvent salt composition, or temperature. (6) When cooperative units for two-state unfolding transitions consist of large fractions of the residues of the proteins, it is possible to relate in a quantitative fashion the enthalpy and entropy changes among different proteins of the same family, *e.g.*, the CGN family, to the sizes of the cooperative units using the concept of an "average nonpolar residue."[5,13] Given only information about the pH and salt dependencies of the transitions for related proteins, it is then possible to predict the temperature of maximum stability for one protein from another even when the reference protein does not itself show a temperature of maximum stability in the available experimental range.

These properties are all consistent with the "force" analysis of Brandts and can be considered to be predictions of that analysis.[3,13,14] As such they provide additional experimental support for this analysis and particularly for the heat-capacity characteristic which Brandts, using data from small solute molecules, was able to establish as a general characteristic of the interaction of nonpolar molecules and groups with liquid water. However, the number and variety of experimental tests of Brandts' analysis are still small and there are sufficient uncertainties about the validity of the two-state analysis in some cases, notably CGN,[14] to require more exhaustive testing of the analysis preferably with proteins of quite different quantitative characteristics. In the chymotrypsinogen family the proteins which we have thus far found to be most useful for additional testing particularly by comparison with CT itself are the dimethionine sulfoxide derivatives of α-chymotrypsin (DMSCT) and CGN.[15] In this article we present the results of studies of the cooperative unfolding process of DMSCT which takes the protein from its "best-folded" state, state A, to an extensively but still incompletely unfolded state, state B. This transition which starts from the "best-folded" state will be called transition I regardless of the specific protein under discussion.[5,6]

Data for the monomethionine sulfoxide derivative (MMSCT), produced from α-chymotrypsin by oxidizing methionine-192 with hydrogen peroxide, are also presented and will be shown to be consistent with the fact that this protein has catalytic parameters for small specific substrates which are essentially identical with those of α-chymotrypsin itself.[16,17] The dimethionine sulfoxide derivative is produced by oxidation of the remaining methionine, MET-180, and has been shown to have significantly different catalytic parameters in the hydrolysis of small specific amide substrates.[17] In a subsequent paper of this series the relation between structure and catalytic efficiency will be presented in detail.

(13) J. Brandts, "Structure and Stability of Biological Macromolecules," S. Timasheff and G. Fasman, Ed., Marcel Dekker, New York, N. Y., 1969, p 65.

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

(15) D. F. Shiao, Ph.D. Dissertation, University of Minnesota, 1968.

(16) H. Schachter and G. H. Dixon, *J. Biol. Chem.*, **239**, 813 (1964).

(17) H. Weiner, C. W. Batt, and D. E. Koshland, Jr., *ibid.*, **241**, 2687 (1966).

Although this and related papers are confined to experimental studies of proteins, it is probable that a number of the important generalizations are immediately applicable to some of the unfolding transitions of both DNA and RNA. Even in those cases in which these transitions are not of the simple two-state type, the processes will tend to be dominated by large heat-capacity changes. Although it was demonstrated some years ago[18,19] that large heat-capacity changes do take place in the unfolding processes of nucleic acids, little attention has been given to this important matter in the interpretation of "melting curves." This omission not only limits the usefulness of such curves but may also make their use quite misleading.

Results

Calculation of Differential Extinction Coefficients. The same changes in indole and phenol spectra used to monitor the A to B transition, transition I, of CT can be used for similar studies of both MMSCT and DMSCT. The spectral data for MMSCT and DMSCT are presented in terms of a differential extinction coefficient at 293 nm, ΔE, defined as the difference in extinction between the protein at a given pH and temperature and the protein in state A at that pH and 25°. However, this quantity could not be directly obtained from the observed spectral changes because, as will be shown, the proteins under consideration were never found completely in state A at any temperature at the pH where experiments were performed. What could be obtained were extinction differences between the protein at a given pH and temperature and the protein in state A at another pH and temperature which served as a reference condition. pH 4.0, 25° was selected as our reference condition since the proteins studied were found to exist completely in state A at this condition. Thus

$$\Delta E\,(\text{obsd}) = \Delta E + \delta E \qquad (1)$$

where δE is the extinction difference between the state A protein at the experimental pH and pH 4.0. δE was estimated to be equal to -0.15 at pH 2.0 and pH 2.5, and equal to -0.10 at pH 3.0 for CT. δE for MMSCT and DMSCT was assumed to be the same as that for CT, and ΔE, as defined, was calculated from eq 1.

Monomethionine Sulfoxide α-Chymotrypsin (MMSCT). For MMSCT the differential extinction coefficient at 293 nm, ΔE, is shown as a function of temperature at pH 2.0 in Figure 1. The temperature dependence of the differential extinction coefficients of the pure A and pure B states, ΔE_A and ΔE_B, is also shown in this figure. The line labeled ΔE_B in Figure 1 was obtained by linear extrapolation of the high-temperature data. As in the case of CT solutions, MMSCT at pH 2.0 always contains some fraction of the protein in state B regardless of temperature. This fact was confirmed by studies of the solubility in high-salt buffer solution. The line labeled ΔE_A in Figure 1 was obtained from spectral data at pH 4.0 where the protein was entirely in state A over the indicated temperature range.

(18) L. G. Bunville, E. P. Geidushek, M. A. Rawitscher, and J. M. Sturtevant, *Biopolymers*, **3**, 213 (1965).

(19) P. L. Privalov, "Water in Biological Systems," L. P. Kayushin, Ed., Consultants Bureau, New York, N. Y., 1969, pp 38–41.

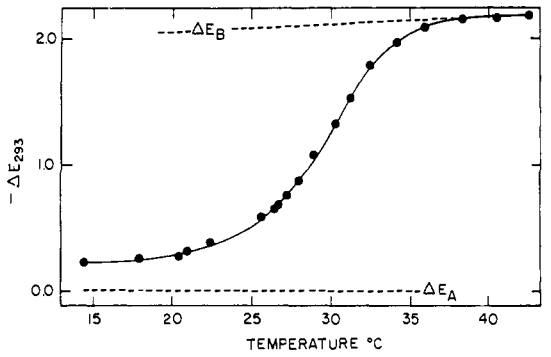

Figure 1. The differential extinction coefficient at 293 nm, ΔE_{293}, of monomethionine sulfoxide chymotrypsin as a function of temperature at pH 2.0. The differential extinction coefficient is defined as the difference in extinction between the protein at a given pH and temperature and the protein in state A at that pH, 25°.

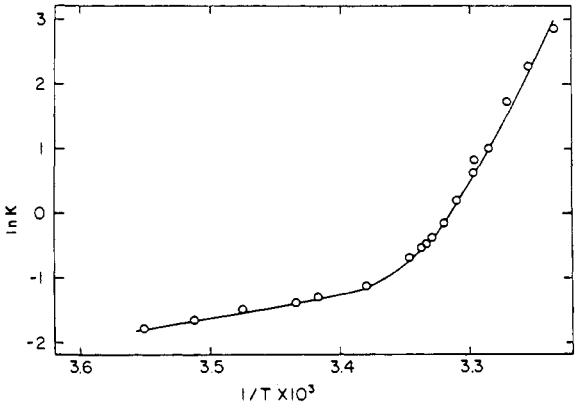

Figure 2. Ln K *vs.* $1/T$ for transition I of monomethionine sulfoxide chymotrypsin at pH 2.0.

Figure 1 was very nearly identical with the corresponding figure obtained with CT. Hence the van't Hoff plot (Figure 2) obtained on the assumption of a two-state mechanism was also essentially identical and transition I for MMSCT can be assumed to be a two-state transition. The apparent standard enthalpy change for the process at several temperatures is compared with the corresponding data for CT in Table I.

Table I. Standard Enthalpy Change for Transition I of Monomethionine Sulfoxide Chymotrypsin and α-Chymotrypsin

Temp, °C	Monomethionine sulfoxide chymotrypsin, kcal/mol	α-Chymotrypsin, kcal/mol
14.0	7	11
28.4	63	67
32.0	86	84

The enthalpy changes are identical within the small errors of the experimental method and form the basis for the conclusions that transition I is identical for the two proteins and that both the states A and B in both protein species are essentially identical.

Dimethionine Sulfoxide α-Chymotrypsin (DMSCT). Initial experiments at acid pH values demonstrated that DMSCT was less stable than α-chymotrypsin. This thermodynamic difference is shown in Table II in

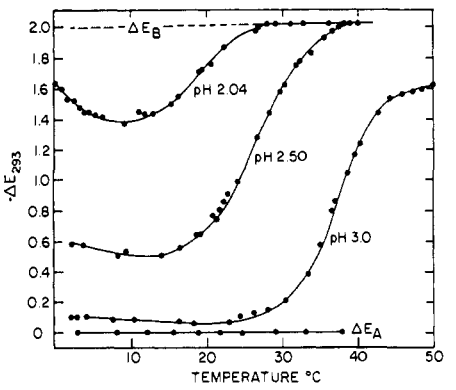

Figure 3. ΔE_{293} of dimethionine sulfoxide chymotrypsin as a function of temperature at several pH values. The pH for each curve is indicated in the figure. The reference condition has been chosen as pH 4.0, 25°.

which the fraction of the protein in state B, determined by loss of solubility in the high-salt buffer, is presented as a function of pH. It can be seen that although both proteins are partially in state B at pH 2 even at 14°, a larger fraction of DMSCT is in this state.

Table II. Fraction Unfolded as a Function of pH at 14° for α-Chymotrypsin and Dimethionine Sulfoxide Chymotrypsin

pH	α-Chymotrypsin	Dimethionine sulfoxide chymotrypsin
3.50	0.00	0.00
3.00	0.00	0.08
2.50	0.00	0.16
2.00	0.05	0.69
1.50	0.13	0.84

The ΔE data for DMSCT are shown in Figure 3. The temperature independence of ΔE_A indicated by the straight line for this quantity in the figure was confirmed by temperature studies at pH 4 under conditions such that the protein was completely in state A. This result is in agreement with similar results obtained with CT. The temperature dependence of ΔE_B was measured at pH 2.0 and 2.5 but could not be determined with high precision at pH 3 because of the rapid aggregation of the protein which occurs in state B above 50°. It was assumed that the straight line obtained at lower pH values could be extrapolated to higher temperatures. It will be noticed that DMSCT is partially in state B even at lowest temperatures at all the pH values studied. An inversion in the sign of the temperature derivative of ΔE is observed at about 10° at pH 2.0 and estimated to be at about 12° at pH 3.0. This observation is consistent with a change in sign in $\Delta H°$ at these temperatures which is confirmed by the fact that the high salt solubility of DMSCT is maximal at about 10° at pH 2.0.

The data of Figure 3 are shown on van't Hoff plots in Figure 4. By comparison of the slopes of these plots at the same temperature but at different pH values it is found that the standard enthalpy of the transition at a fixed temperature is independent of pH. As a consequence all data obtained at fixed values and constant solvent salt composition can be readily adjusted to any single pH value in the experimental range as pre-

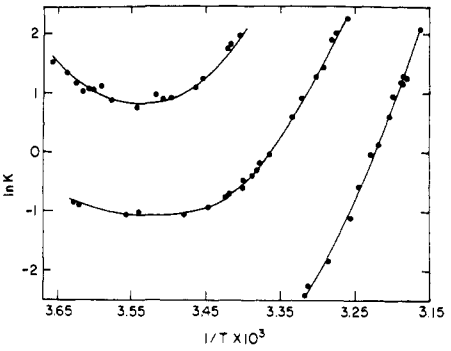

Figure 4. Ln K *vs.* $1/T$ for transition I of dimethionine sulfoxide chymotrypsin at pH 2.04, 2.50, and 3.00.

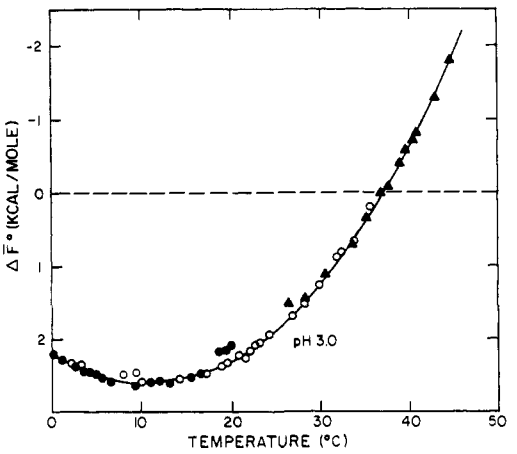

Figure 5. ΔF° *vs.* T for transition I of dimethionine sulfoxide chymotrypsin. The data of Figure 3 have been adjusted to pH 3.0, 0.01 M Cl^- (see text for details). The solid line represents the "best fit" of eq 1 to the data with parameters given in Table III.

viously discussed.[10] Figure 5 is an example of the application of this adjustment. The pH is 3.0 and the salt concentration is 0.01 M chloride ion, there being no added buffer. Brandts' equation, eq 2,[3,13] has been fitted to these data using a modified form[5] given as eq 3.

$$\Delta F^\circ = p(N\Delta h_h^\circ - TN\Delta s_c^\circ + AT + BT^2 + CT^3) \quad (2)$$

$$\Delta F^\circ = \alpha + \beta T + \gamma(T^2 + \delta T^3) \quad (3)$$

In eq 3 δ is the ratio of the coefficient C to B of eq 2 and the least-squares fitting parameters are

$$\alpha = pN\Delta h_h^\circ$$

$$\beta = p(-N\Delta s_c^\circ + A)$$

$$\gamma = pB$$

N is the total number of residues in the protein. The polynomial, $AT + BT^2 + CT^3$, represents the maximal free-energy contribution due to the transfer of the nonpolar side chains from the interior of a protein to an exposed position on the "unfolded" polypeptide. The polynomial is an empirical choice based on consideration of the transfer of nonpolar side chains of amino acids from polar organic solvents, *e.g.*, ethanol, to water, and the amino acid composition of the protein. The quantity p is assumed proportional to the fraction of total residues which experience change in state in the unfolding process and as a result make contributions to the free energy of unfolding, ΔF°. The temperature-independent enthalpy contribution per mole of residues due to factors of stability other than transfer of nonpolar side chains from interior positions to surface positions on the "unfolded" polypeptide is Δh_h° and the corresponding entropy change is Δs_c°.

Comparisons among proteins of the same family can be made without additional assumptions since N, A, B, and C are nearly constant within a given family. In the present instance CT and DMSCT have four fewer residues than CGN out of a total of 245. Undoubtedly the extra residues of CGN introduce quantitative differences between that protein and those members of the family in which the main chain has been cleaved but we shall see that these differences do not appear to have much quantitative significance. Bearing in mind the limitations of the approach we may proceed to compare proteins within a given family by estimating A, B, and C from the component amino acid residues following the method of Brandts.[3,13] These quantities have been calculated previously for CGN and are given in the heading of Table III. The fitting parameters are converted to the quantities p, Δh_h° and Δs_c° of which, according to Brandts, only p should vary in a significant way with the size of the cooperative unit so long as that unit contains a large fraction of the total residues. It is seen in Table III that this is in fact the situation when DMSCT and CGN are compared with CT. Insofar as the constancy of Δh_h° and Δs_c° is not artifactual we may conclude that p is indeed a measure of the relative size of the cooperative unit of transition I of a given protein.

Table III. Thermodynamic Parameters for Transition I at pH 3.00, 0.01 M Cl^- [a]

Protein	P	Δh_h°, cal/mol	Δs_c°, cal/mol deg
Chymotrypsinogen[b]	0.63	800	5.1
α-Chymotrypsin[c]	1.01 ± 0.02	780 ± 10	5.1 ± 0.2
Dimethionine sulfoxide chymotrypsin	0.59 ± 0.04	790 ± 20	5.2 ± 0.4

[a] These parameters have been calculated using values of −2270.2, 18.122, and −0.02792 for A, B and C, respectively. From ref 10. [b] From ref 4. [c] From ref 6.

Similarities of the B States of the Proteins. On the basis of the assumption that the parameter p is a measure of the relative size of the cooperative unit in transition I, an assumption shown to be supported by our data, it may be possible in favorable cases to draw conclusions about the A and B states of proteins which can be tested by other types of experiments. The different values of p found for CGN, CT, and DMSCT indicate that they must have different A states, different B states, or both. Sedimentation data,[12] fluorescence parameters,[20] optical rotatory dispersion,[6,21] and circular dichroism[22] comparisons suggest that the A states are different.[23] The nmr spectra which reflect the

(20) Y. D. Kim, Ph.D. Dissertation, University of Minnesota, 1968.
(21) R. Biltonen, R. Lumry, V. Madison, and H. Parker, *Proc. Nat. Acad. Sci. U. S.*, **54**, 1018 (1965).
(22) H. Parker, R. Lumry, and M. Lund, submitted for publication.
(23) Proton-exchange studies give ambiguous results. Those of Blout, deLoze, and Asadourian[24] suggest differences among the A states

Table IV. Optical Rotatory Dispersion Parameters[a] in States A and B[b]

Protein	State A			State B		
	a_0	b_0	(a_{233})	a_0	b_0	(a_{233})
Chymotrypsinogen	−557	−178	−3500	−587	−185	−3700
α-Chymotrypsin	−404	−175	−3600	−589	−190	−3600
Dimethionine sulfoxide chymotrypsin	−478	−192	−3500	−599	−179	−3500

[a] Moffit–Yang parameters calculated with $\lambda_0 = 202$ nm. [b] State A is defined as the major state at pH 7.0, 25° and state B as the major state at pH 2.0, 60°.

amount and composition of the slower moving, tightly folded material and the loosely held polypeptide undergoing rapid segmental motion show distinct differences in the A states of CT, DMSCT, and CGN.[27,28] On the other hand there are several types of study which suggest that the B states are all very similar despite the fact that CGN has an intact polypeptide chain. The ORD comparisons are given in Table IV and Figure 6.

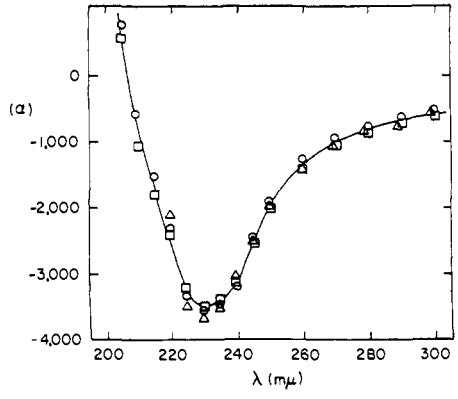

Figure 6. Optical rotatory dispersion patterns for chymotrypsinogen (O-O), chymotrypsin (□-□), and dimethionine sulfoxide chymotrypsin (Δ-Δ) in state B (pH 2.0, 60°).

Although the patterns for all the A states and B states appear to have identical contributions from residues in α-helical conformation, there are distinct differences in the A-state patterns due to varying contributions from additional Cotton effects at about 202 and 228 nm.[6,21] No such differences are observed in state B. The retention of an α-helical ORD pattern corresponding to about 15% of the residues in state B is evidence that the proteins retain some of their state-A structure in state B.

These proteins show typical random-coil ORD patterns in 8 *M* urea.[12] Additional evidence that the states B are very similar is provided by the calorimetric heat-capacity studies of Biltonen, *et al.*,[11] in which it is found that the constant-pressure heat capacities in state B of the three proteins are identical within the errors of the experiment. Thus, although considerable additional conformational freedom might have been expected for CT and DMSCT in state B than for CGN in state B, most available evidence suggests that the differences are not large. If this is the case, the difference in *p* values must be due in largest part to differences in the A states such that DMSCT and CGN are less folded than CT in their A states.

Discussion

The reliability of the estimates of the thermodynamic changes in transition I which we have given depends only on the validity of the two-state approximation. For CT, the validity of this approximation has been established by the three tests: coincidence of the thermodynamic changes estimated by several different observables (test I);[4] monotonic temperature dependence of the apparent standard enthalpy change (test II);[4] and agreement between the apparent standard enthalpy change calculated by the van't Hoff method on assumption of a two-state equilibrium with that obtained calorimetrically (test III).[4] For MMSCT test II supports the application of the two-state approximation but the precise agreement between the transition I behavior of this protein and that of CT provides additional verification. DMSCT also satisfies test II. Test III has been applied to DMSCT at two temperatures by several workers.[11] The calorimetric results are in reasonable agreement with the van't Hoff estimates at 25° providing additional support for the two-state approximation. At 40° considerable experimental variability in the calorimetric results exists, apparently as a consequence of aggregation of the protein at this temperature. The constancy of the Δh_h and Δs_c values (Table III) for the three proteins also provides support for the application of the two-state approximation to CGN and DMSCT since there is little question about the validity of this approximation for CT. Furthermore Brandts[29] finds good agreement between his van't Hoff and his calorimetric enthalpies for CGN as do Biltonen, Schwartz, and Wadsö.[11]

The data of Figure 5 show that Brandts' expression for the standard free energy of unfolding (eq 2 or 3) provides a good fit for the DMSCT transition I data adjusted to pH 3.0 and 0.01 *M* Cl^-. It is also obvious from this figure that the standard heat-capacity change for this process is positive and so large that the temperature dependence of the free energy changes sign in the experimental temperature region. The temperature of maximum stability of DMSCT at pH 3.0 and 0.01 *M* chloride ion is 12°. This is also the temperature of maximum stability predicted at these conditions from chymotrypsinogen data and observed for CT.[10] Although the maximum stability of the A state relative to the B state increases with increasing pH in the acid to

but the more detailed studies of Rosenberg[25] show the A states of CT and CGN to be very similar, a conclusion also supported by the finding of Willumsen.[26]

(24) E. Blout, C. deLoze, and A. Asadourian, *J. Amer. Chem. Soc.*, 83, 1895 (1961).

(25) A. Rosenberg, submitted for publication.

(26) L. Willumsen, *C. R. Trav. Lab. Carlsberg*, 36, 327 (1968).

(27) D. P. Hollis, G. McDonald, and R. L. Biltonen, *Proc. Nat. Acad. Sci. U. S.*, 58, 758 (1967).

(28) R. Biltonen, G. McDonald, and D. P. Hollis, unpublished observations.

(29) W. M. Jackson and J. F. Brandts, *Biochemistry*, 9, 2294 (1970).

neutral range, little variation in the temperature of maximum stability is to be expected on the basis of the Brandts analysis. Indeed if Δh_h° and Δs_c° are constant for a series of proteins of the same family or more specifically for a series of proteins of very nearly identical amino acid sequence the temperature of maximum stability (at constant pH and salt concentration) should be a constant regardless of the size of the cooperative unit. This prediction is consistent with statements made above about the validity of the "average-residue" approximation since the constancy of Δh_h and Δs_c is a consequence of this approximation. It is not necessary that these quantities be correctly estimated by the analysis since constancy requires only that the average residue be essentially identical for the proteins in such a group. We have presented elsewhere[5] some of our reasons for believing that these quantities and p itself have only relative significance as they appear in eq 2 and more recently Shiao has presented evidence that the choice of the temperature dependence made in writing these equations may not be correct.[15] This choice was an arbitrary one in the first place and was based only on the observation that it provided a reasonable fit to the data for the transfer of amino acids from polar organic solvents to water. This quantity is the only one of the three fitting parameters which is determined independently. It is directly related to the heat-capacity change as shown in eq 4 derived from eq 2

$$C_p^\circ = p(2BT + 6CT^2) \qquad (4)$$

$$C_p^\circ = -\gamma(2T + 3\delta T^2) \qquad (4')$$

and thus only to the change in number of nonpolar residue side chains exposed to water in the unfolding process. As a result the p parameter is directly related to the number of average nonpolar residues in the cooperative unit. However, even as p appears in these equations its quantitative significance is uncertain.[5] It will be an incorrect estimate of the total number of residues in the cooperative unit if the fraction of nonpolar residues in the cooperative unit is not equal to the fraction of nonpolar residues in the total protein or if the heat-capacity change per nonpolar residue has not been correctly estimated by the use of model-compound data. We already know that there is a strong tendency for polar residues to be at the outside of the proteins and nonpolar residues to be inside. In small globular proteins charged residues with few exceptions are thus far found at protein–water interfaces. Hence we do not expect the first of these conditions to be met in which case p may very well overestimate the fraction of total protein forming the cooperative unit. Despite these considerations the consistent application of Brandts' analysis should give p values which have relative significance in which case comparison of the p values for CT and DMSCT shows that the cooperative unit for transition I of DMST is about three-fifths of that of CT. Since the p parameters (Table III) of CGN and DMSCT are smaller than that of chymotrypsin, these two proteins should be partially unfolded in state A. These predictions are based on the assumption that state B conformations of the three proteins are thermodynamically very similar, an assumption consistent with calorimetrically determined heat capacities of the three proteins in state B. Nmr studies of CGN and DMSCT[28] show increased segmental flexibility of these two proteins in state A compared to CT. The amount of unfolding of DMSCT in state A compared to the extent of unfolding which occurs during the transition I of CT has been estimated to be approximately 40%, a figure in remarkably good agreement with that based on the results of this thermodynamic analysis. It is interesting to note that the nmr method demonstrates that the unfolded parts of CGN and DMSCT in state A contain few, if any, common residues.[28] This is especially remarkable considering the constancy of Δh_h and Δs_c and suggests that these Δh_h and Δs_c values may also prove to be characteristic of some proteins not members of the chymotrypsinogen A family.

It is reasonable to attempt to associate the altered catalytic parameters of DMSCT with the large unfolded part found in state A of this protein. Nmr studies[28] suggest this unfolded part included His 57 which functions as a base catalyst in the formation and hydrolysis of the acyl-enzyme intermediate in chymotryptic catalysis.[30] It thus seems necessary to assume that specific ester substrates are able to cause at least partial refolding in state A since DMSCT is a good catalyst for these substrates. This problem has been studied using both the nmr method[28] and the thermodynamic and circular dichroism changes in transition I[15] and it is found that refolding does indeed occur. These studies will be described in subsequent publications.

In conclusion we note that despite the limited number of applications of the Brandts analysis which have been reported, there is a remarkable degree of consistency between the quantitative predictions of this analysis and the experimental observations even though eq 2 is probably not the best description of the temperature dependence of the free energy of unfolding. Studies of other members of the CGN family to be published explore the use of different temperature dependencies and demonstrate that internal consistency is retained for all the proteins of this family which have been investigated. Thus although the terms "unfolded" and "folded" have as yet only partial chemical meaning, being primarily defined by the values of experimental quantities, the success of the approach is sufficient to justify more extensive application to other families of proteins. The phenomenological and semi-phenomenological parameters resulting from such studies now provide a means for making quantitative comparisons among proteins, and, as data accumulate and new independent variables are employed to provide additional detail in the analysis, reliable and moderately detailed descriptions of folded states and processes involving changes in these states will develop. This type of approach appears to be the only reliable one available and it should provide the quantitative framework on which to base the interpretation of results obtained using physical methods of all types which will be necessary to provide chemical descriptions. It has already been found that proton-exchange processes of the chymotrypsinogen-A family which depend on transition I are quantitatively consistent with the thermodynamic quantities obtained in our studies.[25] The results of studies of the kinetics of transition I

(30) M. Bender and F. Kézdy, *Annu. Rev. Biochem.*, 34, 49 (1965).

carried out by Pohl[31,32] have also been shown to be consistent with the analysis of Brandts.[5]

Experimental Section

CT (3× crystallized and lyophilized) obtained from Worthington Biochemical Corporation, Freehold N. J., was used without further purification in the preparation of the derivatives. All other chemicals were of reagent grade.

MMSCT was prepared by oxidation of CT with H_2O_2 in aqueous solution at pH 3.0 and 4°. Enzymic activity of this derivative was virtually identical with that of its precursor CT. Chromatography according to the method of Hirs[33] indicated it to be homogeneous and the preparation was used without further purification. Chemical analysis of the preparation by Dr. H. Schachter showed only one methionine residue oxidized to sulfoxide and from previous work[16] this methionine had been identified as residue 180. We are indebted to Dr. Schachter for this analysis.

DMSCT was prepared by oxidation of CT with H_2O_2 in 8 *M* urea, pH 3.0, 4° according to the method of Schachter, *et al.*[16] After dialysis and lyophilization the disulfoxide preparation showed approximately 40% activity toward *N*-acetyl-L-tyrosine ethyl ester, relative to the activity of CT. Chemical analysis of the product by Dr. Harry Schachter demonstrated that 2.0 methionine were oxidized per molecule. Chromatography indicated the product to be better than 90% homogeneous. Examination of the thermal unfolding characteristics gave no indication that the small amount of heterogeneity, probably due to irreversibly denatured and aggregated protein, interfered with the thermodynamic measurements. Hence the preparation was used without further purification. Unless the impurity contributes significantly to the experimental observable as a result of an unfolding transition of its own which overlaps the one under study, it has no effect on the van't Hoff plot. In addition, so long as the process under study is first order in both forward and backward directions, as proved to be the case in this work, uncertainties in the concentration do not lead to uncertainties in the estimates of the entropy and free-energy changes.

The method for determining solubility in high salt buffer used to establish the fraction of protein in state B was a modified form of the procedure described by Eisenberg and Schwert[34] in which the high salt buffer was maintained at pH 3.6 rather than 3.0. This change was necessary for DMSCT because at pH 3.0 there is a significant fraction of state B protein at all temperatures.

Procedures for preparation of the protein solutions, for the difference spectrum measurements, and for establishing the reversibility of the reaction have been described previously.[9,10]

(31) F. Pohl, *Eur. J. Biochem.*, **4**, 373 (1968).
(32) F. Pohl, *ibid.*, 7, 146 (1968).
(33) C. H. W. Hirs, *J. Amer. Chem. Soc.*, 77, 5743 (1955).
(34) M. A. Eisenberg and G. W. Schwert, *J. Gen. Physiol.*, **34**, 583 (1951).

Bromine Catalysis for Carbon Dioxide Hydration and Dehydration and Some Observations Concerning the Mechanism of Carbonic Anhydrase[1]

Michael Caplow

Contribution from the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510. *Received January 10, 1970*

Abstract: The dramatic bromine catalysis of carbon dioxide hydration and dehydration first observed in 1940 has been found to be caused by hypobromous acid. The mechanism for bicarbonate dehydration by HOBr apparently involves a concerted bromination–dehydration reaction and the second-order rate constant for HOBr catalysis is 46 M^{-1} sec^{-1} at 25°; an upper limit equal to 51 M^{-1} sec^{-1} has been set for catalysis of bicarbonate dehydration by elemental bromine. The second-order rate constant for hydronium ion catalyzed dehydration of bicarbonate in 71.5% (v/v) dioxane is increased to 2.5×10^6 M^{-1} sec^{-1} at 25°. It is concluded that the apolar character of the active site of carbonic anhydrase contributes relatively little to the catalytic efficacy of the enzyme but probably contributes to the specific facilitation of the dehydration process as compared with the hydration reaction. A mechanism for the carbonic anhydrase reaction has been proposed in which the metal ion associated with the enzyme acts as a general acid catalyst for bicarbonate dehydration.

A comprehensive interpretation has not yet been provided to account for the dramatic halogen catalysis for carbon dioxide hydration and dehydration demonstrated in 1940.[2] We report here evidence which is consistent with a mechanism for bicarbonate dehydration in which hypobromous acid acts as a brominating agent in a concerted decarboxylation–bromination process. These results as well as those obtained in the study of the influence of an apolar solvent on nonenzymic bicarbonate decarboxylation are applied in considerations of the carbonic anhydrase mechanism.

(1) Supported by grants from the National Institutes of Health (GM-11820) and the National Science Foundation (GB-6802). Address reprint requests to the Department of Biochemistry, University of North Carolina, Chapel Hill, N. C. 27514.
(2) M. Kiese and A. B. Hastings, *J. Biol. Chem.*, **132**, 267 (1940).

Experimental Section

Materials. Oxygen-18 enriched K_2CO_3 was made by heating an ampoule containing an $H_2{}^{18}O$ solution of an equimolar mixture of K_2CO_3 and $KHCO_3$ for 20 min at 100°. The material was taken to dryness at 80° under vacuum after the $KHCO_3$ had been neutralized with base. Bromine solutions were made up fresh daily and concentrations were determined from a molar extinction coefficient at 400 mμ of 139 cm^{-1}. Dioxane was distilled from sodium. Glass-distilled water was used throughout.

Methods. Rates were determined from the isotopic depletion which is associated with the reversible dehydration–hydration of oxygen-18 enriched bicarbonate in unenriched water. Carbon dioxide gas was released from aliquots of the reaction mixture by injecting the sample through a serum stopper into an evacuated tube containing concentrated sulfuric acid. This tube was cooled in a Dry Ice bath until the gas could be transferred to a vessel for introduction into a mass spectrometer (Consolidated Electrodynamics Model 21-614). The per cent oxygen-18 in carbon dioxide was