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Reversible Denaturation of Sperm Whale Myoglobin. I. Dependence on Temperature, pH, and Composition[1]

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Abstract: The denaturation of sperm whale myoglobin as a function of pH and temperature has been studied with optical density measurements in the Soret region (410 mμ) and optical rotation measurements in the ultraviolet (233 mμ). A reversible transition is observed at pH below 5 and above 10. In the pH range from 5.5 to 9 denatured myoglobin precipitates, and when the pH is close to this range irreversible denaturation is very rapid. However, both this material and the precipitate can be largely renatured by an appropriate series of pH and temperature changes. The changes in optical density and optical rotation which accompany the denaturation occur at the same temperature at each pH. Taking the temperature dependence of the optical density and optical rotation of native and denatured myoglobin into account, the fraction of denatured material has been calculated as a function of temperature at each pH. The values calculated on the basis of the two types of measurement coincide. The transition temperatures drop very rapidly as the pH is lowered to 4, and more gradually as the pH is increased to 13. The changes in stability at low pH are attributable to the presence of six buried histidine side chains. Experiments with guanidinated myoglobin indicate a similarly rapid change of transition temperature with pH in the pH range from 4 to 5, but a much smaller change at high pH. This observation indicates that the repulsion between negative charges in native myoglobin contributes to the decrease in stability at high pH. Also, it is probable that the presence of a buried tyrosine side chain does not contribute to this phenomenon, and that this side chain is also abnormal in denatured myoglobin.

Sperm whale myoglobin is one of the few proteins for which a detailed, three-dimensional conformation is known on the basis of X-ray data.[3] Because of this, it is of interest to perform with myoglobin most of the experiments which have typically been done with proteins in order to obtain information regarding their structure. The reversible denaturation of a number of proteins has been studied in detail,[4-9] and several experimental techniques are well established.[8] No comprehensive description of the denaturation of myoglobin has been published hereto. The denaturation of myoglobin at low pH and room temperature has been observed by Theorell and Ehrenberg[10] and has since been investigated in somewhat more detail.[8] The calorimetric measurements of the heat content of myoglobin in solution in the pH range from 2 to 12.5 provided values for the heat of the denaturation reactions at low and at high pH.[11] The calorimetric data were reported to be in agreement with the preliminary results of the work which is to be described in this article.

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(2) Research Career Development Awardee of the U. S. Public Health Service (Grant GM-22015).

(3) J. C. Kendrew, H. C. Watson, B. E. Strandberg, R. E. Dickerson, D. C. Phillips, and V. C. Shore, *Nature*, **190**, 666 (1961).

(4) W. F. Harrington and J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, 30, 21 (1956).

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(6) J. F. Brandts, *ibid.*, **86**, 4291, 4302 (1964); **87**, 2759 (1965).

(7) B. H. Havsteen, B. Labouesse, and G. P. Hess, *ibid.*, **85**, 796 (1963).

(8) J. Hermans, *Methods Biochem. Anal.*, **13**, 81 (1965).

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We have now investigated the denaturation of myoglobin in the pH range from 3 to 13 and at temperatures from 0 to 100° with measurements of optical density and optical rotation. The change in absorption upon denaturation in the Soret band (near 410 mμ) has been observed for myoglobin[10] as well as for hemoglobin.[9] At the most favorable wavelength (408 mμ) the absorption decreases by a factor of four when the bonds between the protein and the heme group are broken (metmyoglobin). Thus, this measurement is a sensitive indication of the state of a limited portion of the molecule.

On the other hand, the optical rotatory dispersion curves of myoglobin should give us information about the conformation of the greater part of the molecule, since this is 70% α helical[3] and possesses a large negative rotation at 233 mμ[12-14] which is greatly decreased upon denaturation.[12]

In the light of the difference between the structural features responsible for the above two properties, a study of the way these two parameters change under identical denaturing conditions should be of special interest in a consideration of whether the denaturation is a cooperative phenomenon or a reaction involving stable intermediates. In the former case, the fraction of denatured material which one may calculate from either parameter must be equal. Unfortunately, these numbers *may* be equal[6] for a noncooperative denaturation as well,[15,16] and a real test of the type of equilibrium present has not yet been performed in the case of the denaturation of *any* protein. Such a test requires the knowledge of other experimental data, such as the molar heat change upon denaturation.[16] However, we believe that we have been able to perform this comparison quite critically in the case of myoglobin. Since the argument is rather lengthy, the pertinent analysis of the experimental data which are described below is the subject of the following companion paper.[17]

Experimental Section

Materials. Crystalline sperm whale myoglobin was obtained from Mann Research Laboratories (Lot. No. 6649). Stock solutions of metmyoglobin were prepared by weighing out a certain amount of protein, dissolving it in distilled water, centrifuging, and bringing to volume with water in volumetric flasks. Glycine and glycine–phosphate buffers were used in the alkaline region; citrate buffer in the acid region. Buffer concentration was between 0.005 and 0.01 *M*. KCl concentration was 0.1 *M* in most solutions. The protein concentration was 0.1 mg/ml. (In a few experiments with solutions twice as concentrated or dilute no significant changes in transition temperature were noted.)

Guanidinated myoglobin was prepared according to an established procedure.[18,19] The number of guanidinated lysine side chains was checked using a Spinco amino acid analyzer kindly put at our disposal by Professor J. Logan Irvin. It was found that less than two unreacted lysine groups remained in the preparations[19] which we used in the denaturation study.

Measurements. Optical density measurements were performed in a Zeiss Model PMQ II spectrophotometer, using 1-cm quartz cells and a Brinkmann thermostated cell holder. In order to avoid the formation of large quantities of irreversibly denatured material during the experiment, we decided not to wait until the system reached a steady state before reading the temperature. Instead, we measured directly the temperature of the solutions under observation by inserting the probe of a YSI Model L2SC tele-thermometer into the reference solution. With this system the temperature could be changed from 25 to 90° in 20 min with the aid of a Haake circulating water bath. Optical rotatory dispersion measurements were performed with a Cary 60 spectropolarimeter. It turned out that it was very important to avoid the use of a metal syringe needle in introducing the solutions into the thermostated cell. Measurements of pH were performed as described.[20]

Results

Metmyoglobin. Typical results obtained when myoglobin solutions of various pH are gradually heated are shown in Figure 1. Both the optical density at 410 mμ and the optical rotation at 233 mμ are seen to decrease relatively abruptly in a given temperature range at each pH. One observes that the rotation of native or denatured myoglobin depends little on pH and somewhat on the temperature. On the other hand, the absorption changes considerably between pH 7 and 10, following the ionization of a proton from the heme–water complex, $pK \sim 9$.[10]

Upon converting these data to the fraction (varying from 0 to 1) of the protein which is denatured at any temperature, it is seen that curves are obtained which are coincident for the absorption and rotation data.

A more complete set of experimental data at low pH is shown in Figure 2. These data have been converted as indicated in Figure 2 for the sake of convenience. One notices here and in Figure 1 a strong dependence of the transition temperature on pH and an accompanying change in slope of the curves. These data are, of course, obtained with buffered myoglobin solutions which are then taken to increasing temperatures. The pH indicated is that measured at 25°. We have also obtained results at constant temperature and varying pH. These are shown in Figure 3. It should be noticed that the pH values reported in Figure 3 have been measured at the temperatures at which the absorption measurements were performed. Furthermore, these data have been corrected as well as possible for the formation of small amounts of irreversibly denatured material (see below).

Reversibility. It is obviously a matter of some importance to establish the reversibility of the observed conformation change, especially in light of the fact that we wish to obtain free energy and enthalpy changes for these reactions from our data.[17] We have accordingly devoted considerable effort to a study of this problem.

In the first place, we have observed that the equilibrium between denatured and native molecules is established quite rapidly when starting with a solution of native protein and changing the pH (at pH below 7) or changing the temperature. These times are not greater than a minute. After the initial rapid equilibration, a further slow increase of the amount of denatured material is observed. Upon reversing the conditions leading to denaturation as soon as the denaturation equilibrium is established the absorption and rotation essentially resume their initial values in a number of experiments. These include: pH reversal at room tempera-

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(13) P. Urnes, K. Imahori, and P. Doty, *Proc. Natl. Acad. Sci. U.S.*, **47**, 1635 (1961).
(14) S. Beychok and E. R. Blout, *J. Mol. Biol.*, **3**, 769 (1961).
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(16) R. Lumry, R. Biltonen, and J. F. Brandts, *ibid.*, **4**, 917 (1966).
(17) J. Hermans and G. Acampora, *J. Am. Chem. Soc.*, **89**, 1547 (1967).
(18) W. L. Hughes, H. A. Saroff, and A. L. Carney, *ibid.*, **71**, 2476 (1949).
(19) L. J. Banaszak, E. H. Eylar, and F. R. N. Gurd, *J. Biol. Chem.*, **238**, 1989 (1963).
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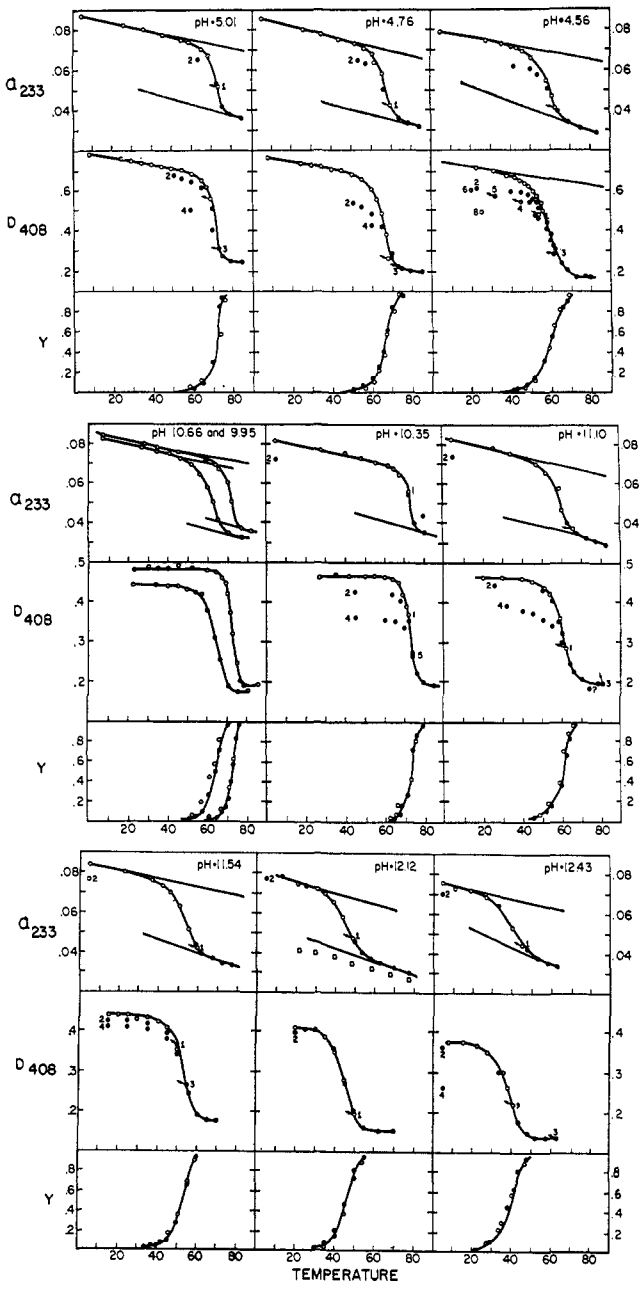

Figure 1. (3 parts) The denaturation of sperm whale myoglobin (concentration = 0.1 mg/ml) in dilute buffered solutions containing 0.1 *M* KCl, followed by measurements of the optical rotation, α, at 233 mμ (top row) and the optical density, D, at 498 mμ (middle row) both measured in 1-cm cells. These data were converted to fraction denatured (y) as a function of temperature, taking into account the temperature dependence of the rotation and absorption of native and denatured myoglobin (bottom row); the extrapolations made when a temperature dependence was noted are indicated by straight lines. Reversibility was studied by lowering the temperature from a point in the transition range (these points are indicated with arrows), the last measurement made before and the first measurement made after lowering the temperature bearing consecutive numbers. This process has in some cases been repeated. The solution at pH 12.12 was subjected to prolonged heating at 80°, after which no increase in D_{408} upon cooling was noted. Optical rotation measurements on this solution are shown (squares) and provide a rationale for assuming a strong temperature dependence of the rotation of denatured myoglobin.

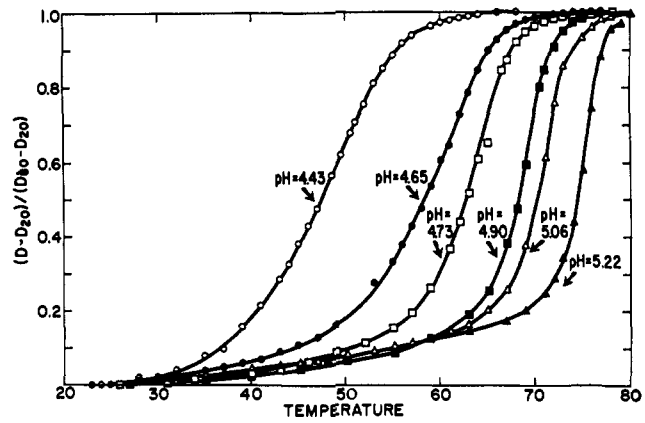

Figure 2. Denaturation of myoglobin followed by measuring the absorption at 410 mμ. The measurements have been plotted as: *the ratio of* the change in absorption with respect to that observed at 20° *to* the value of the change in absorption observed when the solution is taken from 20° to the highest temperature for which the data are shown for each solution. The pH values indicated were measured at 25°. Since these data have not been corrected for the effect of irreversible denaturation, we have not attempted to convert them to the form: fraction denatured as a function of temperature.

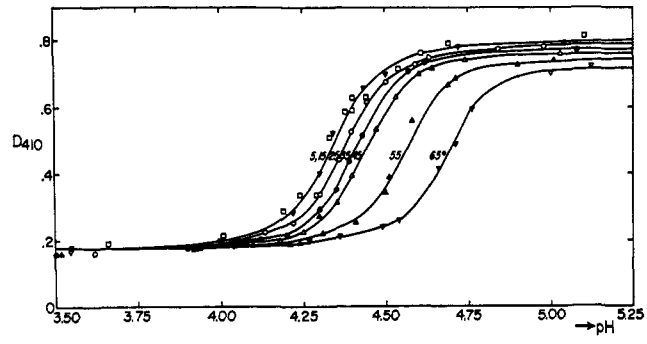

Figure 3. Optical density at 410 mμ of myoglobin solutions at constant temperature as a function of pH. These pH's were measured at the temperature of the absorption measurements.

ture and temperature reversal at pH lower than 5 and greater than 10. Results obtained in the latter manner have been reported in Figure 1. After obtaining the points indicated with arrows, the temperature was lowered and the points obtained marked with a number one higher. The solution was then again heated. Points obtained in each cycle are indicated with different symbols. In a number of experiments, the cooling was repeated when a greater extent of denaturation had been reached.

Considering these results, one may conclude that the data of Figures 1, 2, and 3 do on the whole represent denaturation *equilibria*. Furthermore, in obtaining the data of Figure 3, correction was applied for the formation of small amounts of irreversibly denatured material by following the absorption for several minutes after the pH change and extrapolating the data obtained at times where the reversible denaturation is complete to zero time.

Regarding denaturation in solutions close to the pH range from 5.5 to 9, in which the denatured protein precipitates and reversible denaturation, if at all significant, is not observable for technical reasons (inability to perform or interpret the measurements), we have observed that when such solutions are heated up to about 25% denaturation, the reaction is still largely reversible. However, when the temperature is increased further, one soon reaches a point where upon again lowering the temperature, no increase in amount of

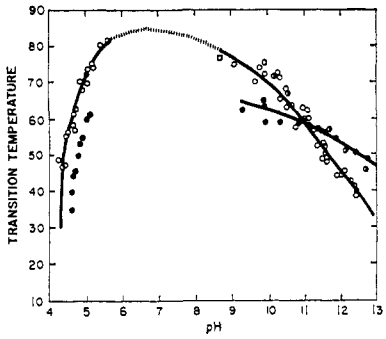

Figure 4. Transition temperatures as a function of pH for metmyoglobin, ○; cyanide metmyoglobin, ◑; and guanidinated metmyoglobin, ●. In the pH range from 5.5 to 9 denatured myoglobin is insoluble, and no experimental data are available.

native material is observed. Apparently, the rate of formation of the irreversibly denatured protein greatly exceeds the rate of formation of the reversibly denatured protein. Considering the results, we have concluded that at these pH's close to the inaccessible range from 5.5 to 9, the observed transition temperatures are somewhat too low and the slopes of the curves considerably too high. This is a caution against the uncritical use of these results for obtaining thermodynamic functions.

Finally, we have performed experiments in which apparently irreversibly denatured myoglobin, both in turbid and in clear solutions, was renatured by lowering (or raising) the pH to the range where the denaturation is largely reversible, and then again bringing the pH at room temperature to a value where native myoglobin is stable. Following this procedure, up to 80% of the absorption or rotation changes observed upon denaturation was found to be reversed.

Myoglobin Derivatives. Experiments with cyanide metmyoglobin at alkaline pH have given slightly higher transition temperatures compared with metmyoglobin. (With cyanide metmyoglobin the greater change in absorption upon denaturation occurs at 420 mμ.)

Guanidinated metmyoglobin has a stability which differs more greatly from that of metmyoglobin. At pH below 11 the transition takes place at a lower temperature, while at pH above 11, the transition temperature is higher than for unmodified myoglobin. Precipitation following denaturation is observed in a wider pH range, and the reversibility is generally poorer.

Transition temperatures for metmyoglobin, cyanide metmyoglobin, and guanidinated metmyoglobin are shown in Figure 4.

Discussion

The description of the reversible denaturation of myoglobin obtained here differs only qualitatively from those given for the denaturation of several other proteins.[5-9] For one, the rather large rate of formation of irreversibly denatured material at intermediate pH is somewhat remarkable. Our results lead us to believe that the product thus formed is not different from the reversibly denatured protein because of the breaking of any covalent bonds, but is the product of aggregation, since it can be renatured by appropriate pH changes. Perhaps the product formed has properties similar to those of the β form of poly-L-lysine[21] which is observed to precipitate very rapidly at high pH when the temperature is brought close to the transition temperature of the helix–coil equilibrium (55°).[21]

In the second place, the good reversibility at high pH is noteworthy in contrast to the lack of reversibility noted when several other proteins are unfolded at high pH. Probably, the absence of disulfide bonds and sulfhydryl groups in myoglobin places it apart from these other proteins in this respect, since disulfide interchange and disulfide bond formation through oxidation with molecular oxygen is expected for denatured proteins at high pH.[22]

Thirdly, it is noted, as with ribonuclease and chymotrypsin, that the stability is greatest at neutral pH. Accompanying the drop in transition temperature, there is a decrease in the steepness of the transition curves which are also noticeably skewed (Figure 2). Furthermore, as with these two proteins, the optical density changes and optical rotation changes characteristic of the denaturation occur in parallel. Also, when the transition curves are plotted as log $[y/(1 - y)]$ *vs.* $1/T$, one observes that the slope of these van't Hoff plots is dependent on temperature, but reasonably independent of pH (although different for solutions at pH <7 and at pH >7). Problems with irreversibility discussed above make the transition data less reliable than, *e.g.*, those obtainable with ribonuclease,[6] and thus the van't Hoff plots obtained by us show more scatter and are not reproduced here.

On the basis of this type of observations, Brandts has concluded[6] that the reversible denaturation of ribonuclease and chymotrypsinogen does not involve *stable* intermediates, *i.e.*, stable types of molecules which are part native and part denatured, even though such forms must, obviously, occur fleetingly during the transition from native to denatured molecules. However, in the present case, it is not necessary to base a conclusion regarding the absence or presence of stable intermediates on this type of analysis alone. By combining the transition data obtained here with the titration data of Breslow and Gurd,[23] one can instead obtain a definitive argument that the transition involves essentially only two states at pH below 7, and probably only two states at pH above 7 as well. This argument is presented in the following paper.[17]

The pH dependence of the transition at low pH is very strong, and is caused by the presence of six buried histidine side chains in the native molecule.[23] These have a very low pK, which becomes normal (~6.5) when the molecule is denatured. As the pH is lowered, the free energy gained by binding protons to these histidines in the denatured form becomes more and more negative, thus decreasing the net free energy of denaturation and the transition temperature.

A similar argument holds, of course, for the net enthalpy of the reaction: at low pH the enthalpy of binding of protons by these histidines must be added to it.[11] The observed enthalpy of denaturation at 25° and pH 4 is found to be close to zero, both on the basis of the negligible temperature dependence of the pH-transition curves (Figure 3) and on the basis of calorimetric measurements.[11] Thus, the enthalpy of denaturation in the absence of proton binding by these

(21) K. Rosenheck and P. Doty, *Proc. Natl. Acad. Sci. U. S.*, 47, 1775 (1961).

(22) *E.g.*, R. Cecil, *Proteins*, 1, 379 (1963).

(23) E. Breslow and F. R. N. Gurd, *J. Biol. Chem.*, 237, 371 (1962).

six histidines is not zero, but, rather, approximately 40 kcal/mole.[11]

Finally, the pH dependence of the transition at high pH should be considered briefly. This dependence is due in large part to the increasing repulsion between charged groups on the molecule as the pH is raised and the negative charge increases. This conclusion follows from the fact that the pH dependence of the transition of guanidinated myoglobin, whose molecules acquire a large negative charge only at pH 12, is considerably smaller. In fact, the pH dependence of the transition of guanidinated myoglobin in the pH range from 10 to 12 is so small that it is probable that the buried tyrosine side chain which is still present in this derivative remains abnormal in denatured guanidinated myoglobin. A similar situation exists in ribonuclease, where one abnormal tyrosine side chain remains abnormal in the reversibly denatured protein.[24] A more precise analysis of this point and several others brought up in this Discussion is presented in the following paper.[17]

(24) J. Hermans and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 3293 (1961).

Reversible Denaturation of Sperm Whale Myoglobin. II. Thermodynamic Analysis[1]

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Abstract: The reversible denaturation of sperm whale myoglobin is analyzed theoretically. The following three criteria are applied to establish that the transition is "two-state" and that no stable intermediates occur. In the first place, the optical density and optical rotation changes accompanying the denaturation parallel one another. This is a necessary, but insufficient criterion. In the second place, the enthalpy calculated from the steepness of the temperature transitions at 30° (van't Hoff enthalpy) is zero at pH 4 and 30 kcalmole at pH 13. This is in agreement with earlier calorimetric values of, respectively, zero and *at least* 30 kcal/mole. However, this information is again insufficient. In the third place, the difference in protons bound per molecule upon denaturation, which is six from titration experiments, is also six when calculated from the steepness of the pH-transition curves. This is a reliable, necessary, and sufficient criterion which allows the exclusion of stable intermediates. This argument is developed further, and molar free energies of denaturation at temperatures from 0 to 65° are calculated by integration of the difference titration curve using the information about the titration of native and denatured myoglobin obtained by Breslow and Gurd and the spectrophotometric measurements of the denaturation equilibrium at low pH. Finally, molar enthalpies calculated from the temperature dependence of the free energy are compared with van't Hoff enthalpies obtained from the temperature transitions at high pH. It is found that these agree well, leading one to conclude that the transition involves no stable intermediates over most of the pH range studied. The following numerical values for the molar thermodynamic functions were obtained at pH 9 and 25°: $\Delta F_{den}^{\circ} = 14.0$ kcal/mole, $\Delta H_{den}^{\circ} = 40$ kcal/mole; $\Delta C_p^{\circ} = 1.4$ kcal/deg mole.

The experimental data on the stability of the myoglobin molecule presented in the preceding paper[3] form, in most respects, a typical equilibrium study of the denaturation of a globular protein and are comparable to results obtained on ribonuclease[4-6] and chymotrypsinogen.[7] However, it is possible to make use of these data in a novel way, owing to particular properties of the myoglobin molecule, and thus to obtain molar free energies of denaturation as a function of temperature. While these have been calculated for the other proteins studied, the procedure followed is subject to the criticism that the usual equilibrium data, obtained by the measurement of optical density, optical rotation, or similar parameters[8] cannot easily be proven to reflect the fraction of molecules which is present in the denatured form (y) and that present in the native form ($1 - y$), as in a two-state transition, but might very well reflect the presence of stable intermediates in which part of the structure is denatured and the remainder native in the same molecule.[9,10] Hence the calculation of equilibrium constants (and molar free energies) on the basis of such data might not be justified.

The latter situation is known to exist in the helix–coil transition of high molecular weight, synthetic polypeptides. For multistate transitions, the molar enthalpy of the reaction: fully native molecule to fully denatured molecule, ΔH_{den}°, is not equal to the value obtained by applying the van't Hoff equation to the equilibrium results. If

$$\Delta F_{den}' = -RT \ln K = -RT \ln [y/(1 - y)] \quad (1)$$

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(2) Research Career Development Awardee of the U. S. Public Health Service (Grant GM-22015).

(3) G. Acampora and J. Hermans, *J. Am. Chem. Soc.*, **89**, 1543 (1967).

(4) W. F. Harrington and J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, 30, 21 (1956).

(5) J. Hermans and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 3283 (1961).

(6) J. F. Brandts, *ibid.*, **87**, 2759 (1965).

(7) J. F. Brandts, *ibid.*, **86**, 4291, 4302 (1964).

(8) See, for example, J. Hermans, *Methods Biochem. Anal.*, **13**, 81 (1965).

(9) D. C. Poland and H. A. Scheraga, *Biopolymers*, **3**, 401 (1965).

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