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]

Jan Hermans, Jr.,[2] and Giuseppe Acampora

Contribution from the Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514. *Received September 9, 1966*

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

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

(1) Supported by a research grant (GM-12175) from the National Institutes of Health, U. S. Public Health Service and research grants (GB-3040 and GB-4577) from the National Science Foundation. Presented at the Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1966.

(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).

where y is the fraction of molecules in the denatured state calculated on the basis of some experimental variable, *supposing* that a two-state equilibrium existed, and if

$$\Delta H_{den}' = -R \text{ d ln } K/\text{d}(1/T) \quad (2)$$

then it is found that

$$|\Delta H_{den}^\circ| > |\Delta H_{den}'| \quad (3)$$

for a transition in which more than two stable states occur.[11] This suggests at once, as a test for determining the importance of stable intermediates, a comparison of molar enthalpies obtained calorimetrically with the enthalpies obtained using the van't Hoff equation. In one extreme case, that in which no stable intermediates occur, the two would be equal. In all other cases, the van't Hoff enthalpy would be smaller than the molar enthalpy (eq 3).[10]

The description of multistate transitions is rather complex. For example, in the theory of the helix–coil transition of infinitely long polypeptides,[12] it is found that $\Delta H_{den}'$, which is the value obtained by applying eq 1 and 2 to, for example, optical rotation data, depends on the enthalpy for the lengthening of the helix by one residue and on the equilibrium constant for initiating a short helix in a randomly coiled molecule. Only at the midpoint of the transition is it found that the behavior of the polypeptide is the same as that of a molecule undergoing denaturation in a single step at

$$y = 0.5, \Delta F_{den}^\circ = 0 \quad (4)$$

We introduce this result here, since it is applied in the analysis described below. Because eq 4 holds for the two extreme cases of two-state and infinitely many state transitions, we shall be assuming that it holds true for the myoglobin transition as well.

Calorimetric data on the denaturation of myoglobin have been obtained at a single temperature of 30°.[13] (The instrument used did not permit work at temperatures much different from room temperature.) Denaturation occurs at this temperature at pH 4.2 and 12.5. The latter pH is so high that only a lower limit of 30 kcal/mole for ΔH_{den}° could be given, information which is useless for a comparison according to eq 3. At pH 4.2, the calorimetric heat of denaturation was found to be equal to zero. This is in agreement with the finding that at this pH and temperature the denaturation equilibrium is temperature independent, *i.e.*, $\Delta H_{den}' = 0$. It is obvious that if $\Delta H_{den}^\circ = 0$ the latter is required by eq 3 whether the transition involves two states or many states.

While it appears to be quite well possible, and probably desirable, to carry out further calorimetric experiments of the same type at higher temperatures with a calorimeter of different design, the same goal of comparing the myoglobin denaturation with a two-state model may be reached by using the pH dependence of the transition curves at constant temperature, rather than the temperature dependence at constant pH. This method is well established, and we shall proceed to describe it only briefly.

Free Energies from Titration Curves. When equilibria between two forms of a molecule are pH dependent, this means that protons are liberated or taken up by the molecule during the reaction. The difference in the number of protons bound per molecule ($\Delta\nu^\circ$) is related to the equilibrium molar free energy and the pH, in very much the same way that the enthalpy is related to the free energy and the temperature

$$\Delta\nu_{den}^\circ = (1/R)\partial(\Delta F_{den}^\circ/T)/\partial \ln a_{H^+} \quad (5)$$

whereas

$$\Delta H_{den}^\circ = \partial(\Delta F_{den}^\circ/T)/\partial(1/T) \quad (6)$$

a_{H^+} being the hydrogen ion activity.[5,14-16] Both equations can be integrated in order to obtain ΔF_{den}°. The integration constant may be determined in one of two ways. Firstly, it may be noted that the hydrogen ion activity or temperature at the midpoint of the transition, $a(1/2)$ or $T(1/2)$, is known and that one may make use of eq 4. Thus

$$\Delta F_{den}^\circ(a_H) = RT \int_{\ln a(1/2)}^{\ln a_H} \Delta\nu_{den}^\circ \text{ d ln } a_{H^+} \quad (7)$$

$$\Delta F_{den}^\circ(T) = T \int_{1/T(1/2)}^{1/T} \Delta H_{den}^\circ \text{ d}(1/T) \quad (8)$$

Secondly, one may perform the integration of the $\Delta\nu^\circ$ (or ΔH°) measured for the reaction: denatured molecules to equilibrium mixture of native and denatured material, choosing one of the limits of integration at a value of a_H (or T) at which the equilibrium mixture consists essentially of denatured material, where $\Delta F^\circ = 0$ by definition, and the other limit in the range where the material is native. (The experimental values of ν or H for the denatured molecules are obtainable by extrapolation, or (for ν) by fast titration methods, as discussed below.) The importance of the parallelism between the parameters ΔH_{den}° and $\Delta\nu_{den}^\circ$ will be clear when it is noted that one can define an apparent difference in protons bound by the equation[17]

$$\Delta\nu_{den}' = \text{d ln } K_{den}/\text{d ln } a_{H^+} = \text{d ln } [y/(1-y)]/\text{d ln } a_{H^+} \quad (9)$$

and that

$$|\Delta\nu_{den}^\circ| \geq |\Delta\nu_{den}'| \quad (10)$$

with the equality holding only for two-state transitions.[11] Thus, eq 10 offers an alternate possibility for the desired comparison, since $\Delta\nu_{den}^\circ$ may be determined by potentiometric titration.

Applicability to Myoglobin

While pH dependence of a denaturation equilibrium implies that there is a difference in the number of protons bound in the two forms of the molecule, in many cases the value of $\Delta\nu_{den}^\circ$ is too small to allow an accurate determination by potentiometric titration. For ex-

(11) One should probably make the reservation here that eq 3 and 10 might perhaps hold for transitions with stable intermediates in which there is an unusual relationship between the enthalpy (or the number of protons bound) and the parameter used to obtain the fraction denatured, y.

(12) B. H. Zimm in "Polyamino Acids, Polypeptides and Proteins," M. A. Stahmann, Ed., University of Wisconsin Press, Madison, Wis., 1962, p 229.

(13) J. Hermans and G. Rialdi, *Biochemistry*, **4**, 1277 (1965).

(14) K. Linderstrom-Lang, *Arch. Biochem.*, **11**, 191 (1946).

(15) F. E. Harris and S. A. Rice, *J. Phys. Chem.*, **58**, 725 (1954).

(16) J. Lebowitz and M. Laskowski, *Biochemistry*, **1**, 1044 (1962).

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

ample, with ribonuclease, one finds experimentally that $\Delta\nu_{den}°$ has a value of about 2.[17] And whereas the titration itself can be performed with good accuracy, a considerable error may be introduced in the process of separating the effects owing to denaturation from that owing to the ionization of groups on the protein molecule which are unaffected by the denaturation reaction. This error may well be a high as 0.5 proton per molecule. It is, therefore, very fortunate that in the acid denaturation of myoglobin, six protons are bound in a very narrow pH interval near pH 4.2[18,19] (Figure 1). Breslow and Gurd showed, furthermore, that these protons are bound to histidine side chains which have an extremely low pK in the native molecule and which are presumably buried in the interior of the molecule. The occurrence of buried histidine side chains is indeed confirmed by the X-ray structure.[20]

Thus, a test of the relationship 10 is immediately possible. The values of $\Delta\nu_{den}'$ obtained at various temperatures from the slopes of the transition curves (Figure 3 of the preceding paper) are given in Table I.

Table I. Number of Protons Bound Accompanying the Denaturation of Sperm Whale Myoglobin

Temp, °C	$\Delta\nu°$, potentiom titration Exptl	Calcd[a]	$\Delta\nu'$, pH dependence of equilibrium[b]	pH at midpoint of transition[b]
5		5.8	5.6	4.34
15		5.7	5.6	4.34
25	6.0[c]	5.6	6.0	4.37
35		5.6	5.8	4.41
45		5.5	5.4	4.44
55		5.1	5.4	4.56
65		5.2	5.6	4.68

[a] See text for information used in calculating these values. [b] Data of Figure 3 of preceding paper.[3] [c] From ref 18.

It is seen that at 25°, the temperature at which Breslow and Gurd showed by titrations that $\Delta\nu° = 6$, $\Delta\nu'$ is also 6. Thus we have at once the conclusion that at this pH and temperature the observed transition can in a very good approximation be described with a two-state model. It is important to notice that this conclusion is reached independently of the assumption of any model to explain the value of $\Delta\nu$, both $\Delta\nu°$ and $\Delta\nu'$ being experimentally determined quantities.

It is seen that $\Delta\nu_{den}'$ is equal to 6 at the lower temperatures, but drops as the temperature increases. Because of this initial constancy of $\Delta\nu_{den}'$ on the one hand and because of the difficulty of performing accurate titrations at those temperatures where $\Delta\nu_{den}'$ is significantly smaller than 6, we have not performed any other potentiometric titrations of myoglobin. Rather, we have *calculated* the titration curves of native and denatured myoglobin using the parameters chosen by Breslow and Gurd to give agreement between theoretical and experimental titration curves at 25°, making certain assumptions about the temperature dependence of these parameters. The first of these assumptions is

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

(19) When $\Delta\nu$ is large, the transition occurs in a narrow pH range by virtue of eq 9. This has the effect of decreasing the absolute value of the error in the determination of $\Delta\nu°$ from the titration curves. Thus the relative error in $\Delta\nu$ becomes extremely small when $\Delta\nu$ is large.

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

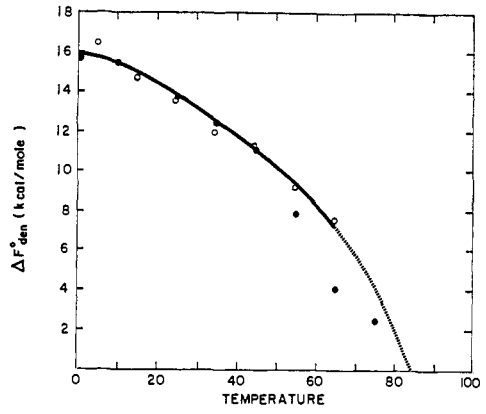

Figure 1. Molar free energy of denaturation of sperm whale myoglobin as a function of temperature at pH 9. Of the two sets of data, those represented by the open circles have been corrected for irreversible denaturation.

that the parameter w, in the Linderstrom-Lang equation

$$pK_{app} = pH - \log[\alpha/(1 - \alpha)] = pK° - 0.87wZ$$

where α is the degree of ionization of any group and Z the average charge on the molecule, does not depend on the temperature for either the native or the denatured molecule. In view of the explicit form of w,[21] this is not unreasonable as long as the shape and size of the molecule remains constant. The latter appears to be a reasonable assumption for the native molecule, but, admittedly, the shape and size of the denatured molecule might vary with temperature and pH. However, the resulting changes in w will not significantly affect the results obtained below. The second assumption regards the temperature dependence of the intrinsic pK, p$K°$, of each type of ionizing group. For the carboxyl groups it is assumed that p$K°$ changes linearly with $1/T$ with a heat of ionization of 1.6 kcal/mole as determined calorimetrically.[13] For the imidazole group it is assumed that p$K°$ differs as much from p$K°$ at 25° as the pK of the imidazole group of β-alanylhistidine. The values used are summarized in Table II.

Table II. Data Used in Calculating Titration Curves of Sperm Whale Myoglobin

Temp, °C	pK_{COOH}[a]	pK_{IM}[b] Native	pK_{IM}[b] Denatured	w_N	w_D	n_{COOH}	n_{IM}, native/denatured
5	4.48	7.04	6.90				
15	4.44	6.81	6.67				
25	4.40[c]	6.62[c]	6.48[c]	0.050[c]	0.034[c]	23[c]	6[c]/12[c]
35	4.36	6.43	6.29				
45	4.32	6.27	6.13				
55	4.29	6.10	5.96				
65	4.26	5.96	5.82				

[a] Calculated assuming a heat of ionization of 1.6 kcal/mole. [b] Calculated assuming a constant difference between these pK's and the pK of the imidazole group of β-alanylhistidine. [c] From ref 18. Values of w were assumed to be the same at all temperatures.

It was then calculated what is the difference between the values of ν for native and denatured molecules at

(21) *E.g.*, C. Tanford, *Advan. Protein Chem.*, 17, 69 (1962).

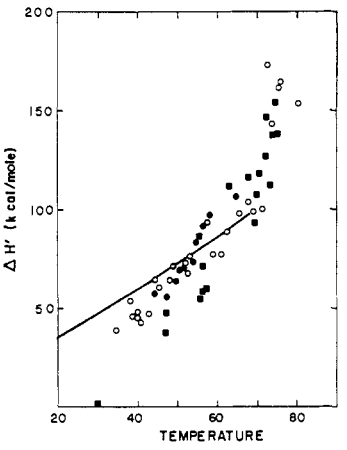

Figure 2. Enthalpy of denaturation of sperm whale myoglobin as a function of temperature. Points shown are for: guanidinated metmyoglobin, ●; metmyoglobin at pH below 8, ■; metmyoglobin at pH above 8, ○. These points were obtained applying the van't Hoff equation to the temperature transition curves. The curve drawn represents the molar enthalpy obtained from the temperature dependence of the molar free energy given in Figure 1.

the pH and temperature at which the midpoint of the transition occurs, *i.e.*, where the $\Delta\nu'$ values were obtained. These numbers are given in the second column of Table I. It turns out that the value of $\Delta\nu_{den}^{\circ}$ is primarily determined by the pH: as the pH comes closer to the pK of imidazole groups, a significant fraction of these groups is dissociated, and, therefore, less protons are bound upon denaturation. It is seen that the values of $\Delta\nu^{\circ}$ and $\Delta\nu'$ agree within 10% at all temperatures.

Free Energy of Denaturation. The comparison of values of $\Delta\nu^{\circ}$ and $\Delta\nu'$ described in the preceding section indicates that the equilibrium between native and denatured forms of myoglobin observed at low pH can be described with a two-state model. However, regardless of the number of states which must be assumed in the model, the values of $\Delta\nu^{\circ}$ as a function of pH can be used to calculate the free energy of denaturation at pH 8–9, using eq 7, as explained above. This can be done quite easily by numerical integration of the difference between the titration curves calculated with the Linderstrøm-Lang equation, or with the integrated form of the equation for the pH-dependent part of the free energy of denaturation[5]

$$\Delta F_{H}^{\circ} = (\Delta F_{h}^{\circ})_{D} - (\Delta F_{h}^{\circ})_{N} \quad (11)$$

$$-(1/RT)\Delta F_{h}^{\circ} = w\nu^{2} + \sum_{i} n_{i} \ln (1 + a_{H}/K_{i}e^{2w\nu}) \quad (12)$$

the subscripts D and N indicating that ΔF_{h}° is to be calculated for the denatured and the native molecule, respectively.[22] Here ΔF_{h}° is the pH-dependent part of the free energy of a protein molecule, w a constant depending on the size and shape of the molecule and ionic strength. The molecule is assumed to contain n_i ionizable groups with ionization constant K_i.

The values of ΔF_{den}° at pH 8–9 so calculated are shown in Figure 1. Two sets of data are shown, the open circles representing the data of Figure 3 of the preceding article,[3] which we consider the most reliable, since in obtaining them corrections were applied for the formation of irreversibly denatured material. The curvature of the line drawn to fit these data is somewhat greater than is warranted by the positions of the points shown. However, there is the added information that at pH 9 the molecule denatures at 85° from which it follows that the free energy of Figure 1 should be zero at this temperature. This observation is the basis for including the dashed portion of the curve in Figure 1.

The enthalpy of denaturation which one calculates from the temperature dependence of the free energy curve of Figure 1, using

$$\Delta H^{\circ} = \Delta F^{\circ} + T\mathrm{d}\Delta F^{\circ}/\mathrm{d}T \quad (13)$$

is, itself, temperature dependent. The enthalpy values calculated in this manner are shown as a solid curve in Figure 2. Here it is important to notice that the calculated values of ΔF_{den}° and ΔH_{den}° do depend on the assumption of the model with six buried histidine side chains. The validity of this assumption is based entirely on the results obtained by Breslow and Gurd and not on any data reported in this and the previous paper. *On the other hand this calculation could have been performed in the same manner for a transition involving stable intermediates, i.e., if we had found that* $\Delta\nu' \neq \Delta\nu^{\circ}$.

It might be asked if for a multistate transition the procedure followed is not uncertain because of the use of eq 4. As has been indicated, the use of this relationship can be avoided entirely by integrating the difference in protons bound measured between the equilibrium mixture of native and denatured molecules and the denatured molecules, over the entire pH range, rather than by integrating $\Delta\nu^{\circ}$ from the pH where $y = 1/2$ to high pH, as done here. Needless to say, the two methods give identical results in this case. In the absence of titration data at temperatures different from 25°, the second method had to be used, unfortunately with its inherent assumptions.

Molar and van't Hoff Enthalpies. Much information may be obtained by comparing the molar enthalpies obtained in this manner with enthalpy values obtained using other methods, in particular by the application of the van't Hoff equation to the equilibrium data obtained spectrophotometrically. The van't Hoff enthalpies taken at the midpoint of each transition curve are given in Figure 2. Open circles indicate values at pH 9 and above, filled squares values at pH 6 and below, while the filled circles represent values for guanidinated myoglobin at pH above 9. It may be added that for those transitions which cover an appreciable temperature range, the curves of fraction denatured as a function of temperature are skewed, in such a way, that the van't Hoff enthalpies calculated at various temperatures for a single solution vary as the $\Delta H'$ values calculated at the midpoints of solutions of different pH reported in Figure 2. Clearly this type of behavior must be followed in a two-state transition,[7] and it was necessary to establish that it is followed here and that there is no inconsistency with our conclusion that the transition is indeed one involving two states.

The data on guanidinated myoglobin are interesting for the following reasons. It is clearly observed that

(22) Equation 12 has here been written in a new form, in that the reference pH at which $\Delta F_{h}^{\circ} = 0$ is the isoelectric point, rather than a very low pH, as in ref 5, and that the changes in free energy are calculated for proton binding, rather than dissociation. The present choice is convenient in this case when the contributions from the heme group and the α-amino group are neglected. (This approximation introduces only insignificant errors.) However, this choice of reference pH may be neither convenient nor correct with other proteins.

they nearly fall on one curve with the data on unmodified myoglobin when plotted as a function of temperature but not when plotted as a function of pH (since the pH dependence of the transition temperature is quite different[3]). It will be seen below that the description of the equilibria obtained by us is in agreement with this behavior.

We may first remark that we have not, unfortunately, been able to measure a transition at pH 8, because of the insolubility of the denatured material. Thus a direct comparison of $\Delta H_{den}'$ at pH 8 and at the transition temperature expected at pH 8 ($\sim$80°, see ref 3) and $\Delta H_{den}°$ at 80° obtained from the data of Figure 2 is impossible. (We have since been able to obtain a reversible transition in this pH range with carboxymethylated myoglobin.[23] The results obtained indicate a value of $\Delta H_{den}°$ of about 70 kcal/mole at a transition temperature of 80°, pH 6.6.[24] However, further experiments with this derivative indicate that these numbers should not be compared directly with the results reported here for unmodified myoglobin.)

In the second place, it should be noted that the description of the low pH denaturation as a two-state equilibrium arrived at above *requires* that the van't Hoff enthalpies at low pH are equal to the molar enthalpies at low pH. The latter are not the same as the molar enthalpies at pH 8, but are lower by an amount

$$\Delta H_{den}°(\text{pH 8}) - \Delta H_{den}°(\text{low pH}) = -\sum_i \Delta\nu_{den,i}°\Delta H_i°$$

the subscript i indicating the ionizing group of type i and $\Delta H_i°$ its heat of ionization. In practice, this means that at low temperatures the difference in enthalpy corresponds to the enthalpy of binding protons to six histidine side chains or about 40 kcal/mole. It will be noted that this behavior is observed within the accuracy of the experiments.

In the third place, it is seen that the molar enthalpy at pH 8 and the van't Hoff enthalpy at pH above 9 are the same at temperatures between 40 and 60°. (It will be realized that we are here looking at data which we have not heretofore considered in this discussion.) On the basis of this observation, we think that the molar enthalpies of denaturation at high pH are equal to those at pH 9 (no large heat effects of proton dissociation upon denaturation being expected in this case) and that, therefore, the equilibrium observed at high pH can be adequately described with a two-state model in the range from 40 to 70°. At lower temperatures, the data would indicate that perhaps this conclusion does not hold any longer as exactly. (However, here the transition curves are, of course, those at highest pH, and these are being compared with data obtained on the basis of experiments at quite low pH.) As far as the transitions at pH above 9 and temperatures greater than 70° are concerned, we think that the large value of $\Delta H'$ is caused by the occurrence of irreversible denaturation and that a comparison of these van't Hoff enthalpies with the values expected on the basis of the extrapolation of the curve of the molar enthalpy shown in Figure 2 is not possible.

Finally, an extension of these conclusions to include the denaturation equilibria of guanidinated myoglobin would appear very reasonable in the light of the observed behavior of this derivative. In fact, the observations made with guanidinated myoglobin allow one to make an independent check of the analysis presented. Since guanidinated myoglobin retains the six buried histidine side chains,[25] the calculation of the free energy of denaturation at pH 8 from the transition data at low pH proceeds in the same way as with unmodified myoglobin. One finds that the guanidinated product is less stable by 4000 cal/mole. If the temperature dependence of the free energy of denaturation is the same as for native myoglobin at all temperatures, one can easily calculate the transition temperature corresponding to the new value of the free energy of denaturation to be about 70°. Insolubility of the denatured protein again makes direct observation of the transition at pH 8 impossible. However, from the data at high and low pH, one would obtain an approximate transition temperature of about this magnitude at neutrality.

Conclusion

We have seen that the pH dependence of the transition at low pH is readily accounted for by the presence of six buried histidine side chains. On the other hand, we have no such ready explanation for the pH dependence of the transition at high pH. Two effects which might contribute to the lowering of T_{tr} are the increased mutual repulsion by the negative charges as the pH is raised and the tyrosine and lysine side chains dissociate a hydrogen ion, and the presence of a buried tyrosine side chain in the native molecule.[26] On the basis of the small pH dependence of T_{tr} for guanidinated myoglobin at high pH, one would conclude that the second effect does not occur, and, hence, that the abnormal tyrosine side chain is abnormal in the reversibly denatured molecule as well. The occurrence of an abnormal side chain in reversibly denatured ribonuclease has been noted before.[17] It follows that it is premature to assume that the reversibly denatured molecule observed here is a completely unfolded polypeptide chain. The same conclusion might be reached on the basis of the observation that under denaturing conditions the reversibly denatured material is slowly converted to a material which does go back to the native conformation even more slowly, but which may be renatured with an appropriate temperature–pH cycle.[3] These observations suggest that unfolding beyond the reversibly denatured form described here does occur even though this process is not accompanied by noticeable changes in optical density or optical rotation.

There has been some discussion in the literature recently about the possibility of the occurrence of two-state transitions in proteins.[6,7,9,10] In these articles both theoretical arguments and experimental data were produced. Obviously, the data on myoglobin analyzed here can tell one very little about the correct way of interpreting the data on ribonuclease and chymotrypsinogen quoted. On the other hand, the possibility of the occurrence of a two-state equilibrium in protein denaturation is now well established, with the reservation that the denatured form observed is perhaps not the final product of the reversible denaturation. Still,

(23) L. J. Banaszak, P. A. Andrews, J. W. Burgner, E. H. Eylar, and F. R. N. Gurd, *J. Biol. Chem.*, **238**, 3307 (1963).

(24) D. Lohr, G. Acampora, and J. Hermans, unpublished results.

(25) L. J. Banaszak, E. H. Eylar, and F. R. N. Gurd, *J. Biol. Chem.*, **238**, 1989.

(26) J. Hermans, *Biochemistry*, **1**, 193 (1962).

the enthalpy associated with the step observed here is quite large, and, furthermore, the optical rotation indicates the loss of most of the α-helical structure which is taken up by 70% of the chain in the native molecule. Hence, a very significant structural change has been shown to occur cooperatively within a narrow limit.

Because of the uncertainty about the conformation of the product of reversible denaturation of myoglobin, it would appear premature to interpret the measured values of the free energy or the enthalpy in terms of the ums of contributions from local interactions, as suggested by various authors.[7,27,28] However, it is interesting to notice that the calculated molar specific heat of denaturation of about 1400 cal/mole degree is qualitatively in agreement with the similarly large values which Brandts had to assume in analyzing the denaturation equilibria of chymotrypsinogen and ribonuclease as two-state equilibria.[6,7] Rather than make the assumption of drastic changes in the structure of the denatured molecule with temperature we also would explain this observation by pointing to the observed large specific heat of solution of hydrocarbons in water, which should correspond to a large specific heat in those molecules in which the hydrocarbon side chains are exposed to the aqueous solvent, *i.e.*, the denatured molecules.[27]

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Electron-Transfer Characteristics of the Prosthetic Group of Hemoproteins[1,2]

Theodore Mark Bednarski and Joseph Jordan

Contribution from the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received October 26, 1966*

Abstract: Diffusion-controlled polarograms corresponding to the ferroheme–ferriheme electron transfer were obtained at the dropping mercury electrode, in buffered aqueous solutions in a range of ionic strengths between 0.1 and 0.2. Experimental conditions were judiciously controlled to eliminate spurious inferences due to electric double layer and interfacial adsorption phenomena. Remarkably, the ferroheme–ferriheme electrode reaction proved to be *a two-electron-transfer process, which proceeded with Nernstian reversibility from pH 7 to 13.* The Fe(III) form of the electroreactive species was invariably dimeric. The electroreactive ferroheme species was monomeric at $7 < pH < 9$ and at $pH > 12.5$, and dimeric in the intermediate alkalinity domain. Thus the conceptually simplest possible two-electron transfer, *viz.*, Fe(III) dimer + 2e ⇄ Fe(II) dimer, prevailed when $10^{-5} < [OH^-] < 3 \times 10^{-2}$. In contradistinction "dimer–monomer cleavage," *viz.*, Fe(III) dimer + 2e ⇄ 2Fe(II) monomers, occurred at both higher and lower alkalinities. Unequivocal assignments are offered identifying ligand groups at five octahedral coordination positions of each of the two iron centers in the electroreactive dimers. The two iron–porphyrin building stones of the dimers were linked *via* the remaining coordination sites. Variations in the nature of this "dimerization bond" (by propionate or water bridging) may account for the significantly different Stokes radii of the ferriheme dimers observed at low and high pH.

Empirical observations have consistently suggested the prevalence of a systematic parallelism between electron-transfer processes in homogeneous solution (isotopic self-exchange: *e.g.*, $*Fe^{2+} + Fe^{3+} = Fe^{2+} + *Fe^{3+}$; cross reactions: *e.g.*, $Fe^{2+} + Ce^{4+} = Fe^{3+} + Ce^{3+}$) and electrode reactions occurring at a heterogeneous phase interface. Recent theoretical developments[3] have rationalized these findings in terms of mathematical expressions which correlate the kinetics of self-exchange and cross and electrode reactions. Thus, the electrochemical characteristics of a given redox couple can indeed provide valid clues on electron donor–acceptor behavior in solutions and *in vivo*. On the basis of these considerations, a reinvestigation of the electrochemical behavior of the ferroheme–ferriheme system was undertaken in these laboratories, under judiciously controlled "model conditions," which were expected to yield insights into the remarkably paradoxical electron-transfer properties of hemoglobin.[4]

The iron–protoporphyrin system has been previously studied in aqueous solutions by prominent investigators.[5–7] using classical potentiometric and spectrophotometric methods; their results lead to a significant controversy concerning the involvement of polymeric electron donor–acceptor species. Similar findings were reported by Brdicka and Wiesner[8] on the basis of a brief polarographic investigation. In contradistinction, recent papers on metal porphyrins in nonaqueous solvents revealed straightforward electrochemical be-

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