Heat of Ionization and Denaturation of Sperm-Whale Myoglobin Determined with a Microcalorimeter*

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ABSTRACT: A Calvet microcalorimeter has been used to measure the heat effects of mixing dilute neutral sperm-whale myoglobin solutions with dilute acid or base. Values of the enthalpy as a function of *p*H were obtained in the *p*H range from 2 to 12.5. On the basis of prior knowledge of the ionization of the various side chains in the myoglobin molecule and of the normal heats of ionization of these groups, the data can be accounted for if corrections are applied for the heat of denaturation.

For the acid denaturation (*p*H $\sim$ 4.5), a heat of approximately 40 kcal/mole has to be assumed, which precisely balances the heat of uptake of hydrogen ions by the six histidine side chains which are abnormal in the native protein. Further, there occurs denaturation at *p*H > 11.5, which is accompanied by a heat uptake of at least 30 kcal/mole. An upper limit to this heat of denaturation could not be established, since the measurements could not be extended to a high enough *p*H. It is pointed out that these results are in agreement with the results of spectrophotometric determinations of the unfolding equilibrium.

The determination of values of thermodynamic parameters for ionization and, especially, for conformational changes of proteins is of increasing interest, as insight is gained into the nature of the forces responsible for the stability of the structure of native protein molecules (Kauzmann, 1954, 1959), and as theories aiming at a quantitative description of these interactions are developed (Laskowski and Scheraga, 1954; Némethy and Scheraga, 1962) and model compound systems are studied which approximate the behavior of proteins in solution (Tanford, 1962a). So far, free energies of denaturation and ionization have been determined by the measurement of equilibrium constants—of ionization (Cohn and Edsall, 1943) and of denaturation (as discussed by Hermans, 1965). The corresponding enthalpies were obtained by studying the temperature dependence of these equilibrium constants and applying the van't Hoff relation. Since these results existed for comparison, it was of interest to measure the enthalpies directly using a microcalorimeter.

The Calvet calorimeter available for this study permits measurements at constant temperature and allows accurate determination of small reaction heats (Calvet and Prat, 1956). Specific heats cannot be determined accurately. Thus denaturation must be brought about by changing the solvent, the solvent change chosen here being a change in *p*H.

The protein chosen for this study was sperm-whale myoglobin. Its use has the advantage that a complete knowledge of its structure (Kendrew *et al.*, 1961) and amino acid sequence can be relied on (Edmundson and Hirs, 1961). Also, myoglobin denatures at low *p*H (Theorell and Ehrenberg, 1951) and it is thus a suitable object for this study. Finally, the potentiometric titration curve of sperm-whale myoglobin has been carefully investigated by Breslow and Gurd (1962). It will be seen here that a knowledge and understanding of this curve is a prerequisite for an interpretation of data of the enthalpy as a function of *p*H.

Experimental

Materials. Crystalline sperm-whale myoglobin was obtained from Seravac Laboratories, Colnbrook, England (lot 2).

Instrument. The instrument used was a Calvet microcalorimeter, built by Société D.A.M., Lyon, France. It consists of a thermostated block with two cavities. The cavities contain silver tubes which are in thermal contact with the block, each via 1000 thermocouples, connected in series. In these silver tubes, the stainless steel cells (*vide infra*), one the sample, the other the reference, are introduced. The two thermocouple piles are connected so that they oppose each other, and the circuit is closed with a recording galvanometer. It can be shown (Calvet and Prat, 1956) that the integral of the current as a function of the time is proportional to the difference in heat produced in the two cells, and calibration can be performed by producing a known quantity of heat electrically in the sample cell. A further description of the technique and of this particular instrument can be found in Calvet and Prat's (1956) text. (A similar instrument and its use is described by Kitzinger and Benzinger, 1960.)

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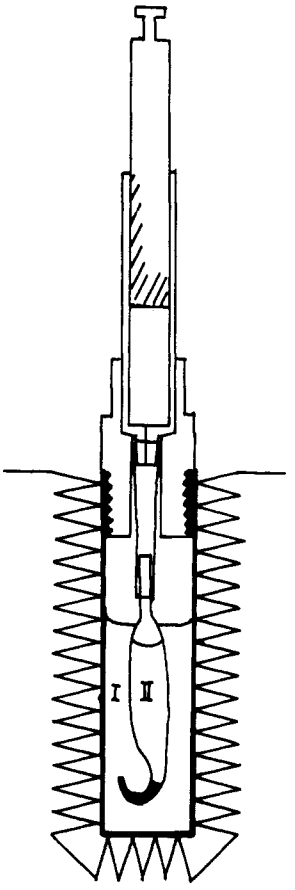

FIGURE 1: Schematic diagram of the cell used for the calorimetric measurement. It is described in the text.

Cell Design. The design of the cell used is adapted to the particular experiment to be performed. The basic cell is a hollow stainless steel cylinder 2 cm in diameter and about 10 cm long. Teflon is preferred for the screwed-on stopper. In this study we pierced the stopper (Figure 1) so that a 1-ml tuberculin syringe fitted into it and could be connected via a short piece of Teflon tubing to a small glass tube containing acid or base (II in Figure 1). Compression of the syringe forces out the mercury or (in later experiments) the vaseline which seals the end of this tube during thermal equilibration, and permits mixing of the acid or base with the dilute neutral myoglobin solution (I). To achieve complete homogeneity, the piston was moved up twice more and then left in the down position. An outlet for air through the Teflon stopper was provided (not shown in Figure 1. Although this work was completed using this arrangement, it is now felt that it is not ideal, since a blank measurement, when mixing water with water, gives a small heat absorption, probably owing to evaporation of water into the air pulled in when the syringe piston is moved up. Although this heat effect is rather small compared with those measured for the myoglobin solutions, it could be much smaller, and this is to be preferred).

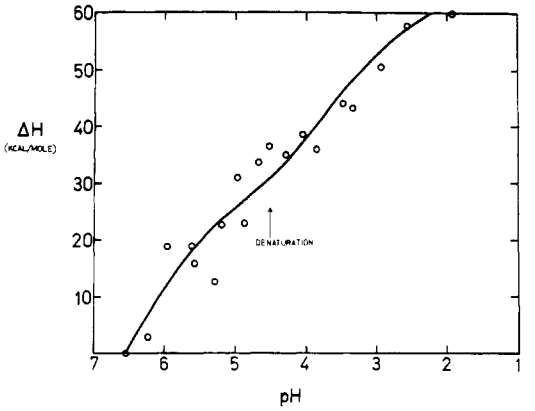

FIGURE 2: Molar heat evolved when dilute neutral myoglobin solutions are acidified with dilute HCl. Curve is theoretical. The *p*H at which the protein is 50% denatured is indicated.

Measurements. The measurements were made at 30° using about 20 mg of myoglobin dissolved in about 6 ml of previously boiled (i.e., CO_2-free) 0.15 N KCl for each experiment. The measurements require from 1 to 2 hours to achieve thermal equilibrium and another 45 minutes to again reach thermal equilibrium after the mixing of the two solutions. As soon as possible after the measurement, the solutions were taken out of the sample cell and their *p*H was measured at 30° with a Radiometer Model 4 *p*H meter, using a B-type electrode, standardized according to Bates (1954).

The area of the curves obtained was measured with an accurate planimeter, and the corresponding heat evolved or absorbed was calculated using previous calibration data corrected for the blank reaction. (It was found that dilution with water of HCl solutions of concentration 0.1 N and higher and of KOH solutions of concentrations 0.5 N and higher produced amounts of heat which were significant corrections to those measured when the same amounts were mixed with myoglobin solutions.) The corrected heats were then converted to molar values using the known amount of myoglobin used in the experiment and the mw 17,800.

Results

*Low p*H. The results at low *p*H are reported in Figure 2 as the heat evolved accompanying the binding of H^+ ions to myoglobin upon lowering the *p*H. It is seen that the reproducibility is reasonably good.

*High p*H. The results obtained upon adding base are shown in Figure 3. It is seen that the precision is good below but not above *p*H 10.5. Measurements made starting at *p*H 9.5 with a myoglobin solution made alkaline by the addition of KOH gave consistent results, indicated by the solid circles. (It should be noted that of these points the one at *p*H 9.5 was adjusted to best agree with the other data.) Since the erratic data fall below the curve drawn through the solid circles, it is our explana-

TABLE I: Parameters Describing the Ionization of Sperm-Whale Myoglobin.[a]

Type of Group	$n_i°$	$pK_i°$		$\Delta H_i°$ (kcal/mole)	
		Native	Denatured	Normal Range[b]	This Work
Carboxyl	24	4.40[c]	4.40[c]	0–2	1.6[d]
Histidine	6	6.62[c]	6.48[c]	6–8	7.1
Histidine	6	Very low[c]	6.48[c]		[e]
α-Amino	1	7.8[c]		11	(11)
Hemic acid	1	8.9[c]		6	(6)
Tyrosine	2	10.0[f]		6–8	6.1[g]
Tyrosine	1	Very high[f]			[e]
Lysine	19	10.0[h]		13–14	12.7[g]
Arginine	4	12[b]		>14	[e]
w		0.050[c]	0.034[c]		

[a] Ionic strength 0.15 M. [b] Taken from Tanford (1962b); see also Cohn and Edsall (1943). [c] From Breslow and Gurd (1962). [d] Breslow and Gurd (1962) have calculated an average value of 2.1 kcal/mole for the average heat of ionization in the carboxyl region by studying the temperature dependence of the titration curve. Their analysis does not take into account possible changes in the electrostatic interaction factor with temperature, and some difference with the true $\Delta H_1°$ for carboxyl groups is therefore expected. [e] Not determined. [f] From Hermans (1962). [g] See text. [h] Unpublished observations made by E. Breslow and also by J. Hermans.

tion that in these experiments leakage occurred during the thermal equilibration. The strength of the base added to the solutions increases rapidly as the *p*H to be reached is 10 or higher. A minor leakage of strong base into the solution could cause a *p*H change of one or two units during the thermal equilibration period. Clearly, the accompanying heat evolution would no longer be measured after mixing of base and solution, and the determined heat effect would be too low. (For some reason such leakage apparently did not occur when the little tube contained strong acid.)

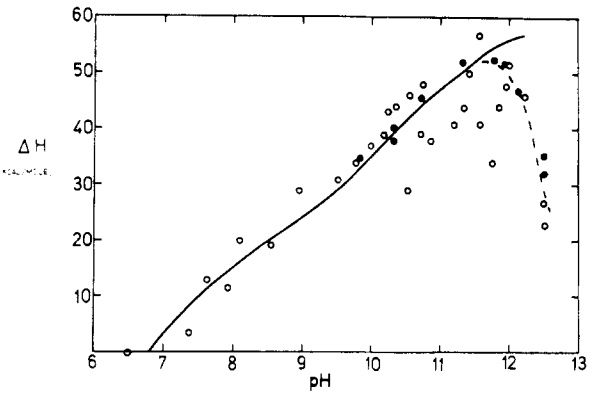

FIGURE 3: Molar heat evolved when dilute neutral myoglobin solutions are made basic with dilute KOH (open circles). The filled circles indicate measurements made starting with a myoglobin solution of *p*H 9.5. The point at *p*H 9.5 has been adjusted to coincide with the other data. The drawn curve is theoretical.

Discussion

In order to interpret the data obtained, the heat effect of the changes in ionization of the amino acid side chains must be separated from those accompanying possible denaturation. Thus, one begins by investigating to what extent the experimental heat is compatible with that calculated by summing the product of the degree of ionization and the "normal" heat of ionization of all the individual side chains:

$$\Delta H_{\text{ion}}° = \sum_i n_i \Delta H_i° \qquad (1)$$

where n_i is the number of groups of type i ionized out of a total of $n_i°$, and $\Delta H_i°$ is the value for the heat of ionization observed in model compounds and apparently normally behaving proteins.

Here n_i is related to the *p*H by the Linderstrøm-Lang approximation (e.g., Tanford, 1962b).

$$p\text{H} - \log [n_i/(n_i° - n_i)] = pK_i° - 0.87wZ \qquad (2)$$

where $pK_i°$ is the intrinsic or normal pK of this type of side chain, w a constant depending on the size and shape of the protein molecule and ionic strength, and Z the charge of the molecule. The best values of the $pK_i°$ and w are determined by fitting a theoretical titration curve given by the sum of all n_i values to the points determined experimentally.

Breslow and Gurd (1962) have done this work for sperm-whale myoglobin and have obtained $pK°$ values for the carboxyl group and six of the twelve histidine side chains (Table I). The other six histidine side chains

titrate normally only in denatured myoglobin, and in the titration of native myoglobin take up protons around *p*H 4.5 where denaturation occurs. Two of the three tyrosine side chains and all the lysine side chains are found to titrate reversibly in the native protein, with normal $pK°$, except for an unexplained steepening of both these titrations in the *p*H range from 11 to 12. (Hermans, 1962 and unpublished; E. Breslow, unpublished).[1]

This information has been collected in Table I, as have the normal values for the heat of ionization of these side chains (after Tanford, 1962b). Before computing a theoretical curve using equation (1), however, it should be realized that it is clear from inspection that the sharp step in the titration curve, which occurs at *p*H 4.5 and which corresponds to the binding of hydrogen ions by the six abnormal histidine side chains upon denaturation, is not paralleled by any step of more than a few kcal/mole in the enthalpy curve of Figure 2. Yet, according to Table I, a step of *at least* 6 × 6 (i.e., of 36) kcal/mole is expected. It is to be concluded that the "missing" heat is absorbed in the unfolding of the myoglobin molecule from the native to the denatured state. The heat of denaturation is equal and of opposite sign to the heat of binding of protons by the six abnormal histidine side chains, i.e., is equal to approximately 40 kcal/mole.[2] It is seen here how information about the denaturation reaction can be obtained by combining information from the titration curve with data of the enthalpy as a function of *p*H. This is discussed further in succeeding paragraphs.

The value of 40 kcal/mole for the heat of denaturation of myoglobin is comparable to that for ribonuclease (Harrington and Schellman, 1956; Hermans and Scheraga, 1961). The absence of any sizable heat effect upon denaturation at *p*H 4.5, furthermore, is supported by a study of the low *p*H denaturation equilibrium at three different temperatures, which also showed that the *net* heat of denaturation is zero (Hermans, 1965).

We may now fit a theoretical curve to the experimental data, neglecting the ionization of the six abnormal histidines. The best fit was obtained with the curve drawn in Figure 2, using the $\Delta H_i°$ values shown in the last column of Table I.

The data obtained by adding dilute KOH to the myoglobin solutions (Figure 3) can be interpreted in much the same way by using, instead of equation (1), the following expression:

$$\Delta H_{\text{ion}}° = \sum_i \Delta n_i \, (\Delta H_i° - 13.3 \text{ kcal/mole}) \qquad (3)$$

which reflects the fact that the hydrogen ions are neutralized upon dissociation from the protein. Good agreement between theory and experiment is obtained using the $\Delta H_i°$ values of Table I (Figure 3). In the alkaline range different types of groups ionize in strongly overlapping ranges, and thus some of the $\Delta H_i°$ values cannot be determined accurately. With respect to the values listed in Table I, it should be noted that reasonable agreement is obtained using the most acceptable values of $\Delta H_i°$ for the α-amino group and for hemic acid, the latter value following from the work of George and Hanania (1952). Further, the (near) equality of the $pK°$ values of tyrosine and lysine makes a separation of their contributions impossible. The value of 6.1 kcal/mole for tyrosine side chains followed from a study of the temperature dependence of the spectrophotometric titration curve of guanidinated myoglobin, which does not show the steepening between *p*H 11 and 12 observed with the native protein (J. Hermans, unpublished). Using this value, about one-half the difference in enthalpy in going from *p*H 9 to 12 is accounted for; the remainder of about 12 kcal/mole can be used to calculate $\Delta H_i°$ for the lysine side chains using equation (3).

Clearly, the data at *p*H 12 and higher indicate the occurrence of a conformational change, involving heat uptake of at least 30 kcal/mole.[3] Unfortunately, the data could not be extended into the *p*H range where the experimental heat no longer decreases with *p*H, because of the rapidly increasing blank correction. However, the heat taken up at *p*H 12.5 is certainly not much smaller (and perhaps larger) than that taken up for *denaturation* at *p*H 4.5.

It is interesting to note that optical density measurements at 410 mμ and optical rotation measurements at 230 mμ also indicate that myoglobin unfolds (reversibly) at *p*H 12 and higher at this ionic strength (0.15 M). Furthermore, there is evidence from the same type of measurements that sperm-whale myoglobin unfolds reversibly at temperatures of 90° and higher, at *p*H 8. From the temperature dependence of this (at least initially) reversible reaction, a transition temperature of 120° and a heat of denaturation of approximately 30 kcal/mole may be deduced (J. Hermans, unpublished). The three values of $\Delta H_{\text{den}}°$ are thus quite consistent with one another.

The experimental values of $\Delta H_i°$ listed in Table I bear out those measured for model compounds and other proteins. As was pointed out in the introduction, the literature values were obtained from the temperature dependence of the ionization constants, using the van't Hoff relationship, and not calorimetrically. Reliable values for the heats of ionization of normal side chains are therefore available. Having a prior knowledge of the titration curve and a proper analysis of this curve using the Linderstrøm-Lang equation, one can use these values to calculate a theoretical curve of enthalpy versus *p*H for any protein. Differences between the calculated curve and the observed data

[1] The authors are indebted to Dr. Breslow for discussing these data with them.

[2] The heat of transfer of these imidazoles to the solvent is to be considered a part of the heat of denaturation. Hence, the heat effect balancing that of the denaturation is that of the binding of protons by six perfectly normal histidine side chains.

[3] The arginine side chains are not expected to ionize appreciably below *p*H 13, because of the electrostatic repulsion.

will give pertinent information about other reactions taking place in the protein molecule, denaturation being the most common example. The enthalpy of denaturation can be calculated quite easily once these procedures have been followed.

The heat of denaturation thus determined may subsequently be compared with values obtained from the temperature dependence of the denaturation equilibrium, which can nearly always be studied by other techniques, for example, with spectrophotometric measurements or with measurements of the optical rotation. The two methods of obtaining ΔH_{den}° need not give identical results, as they apparently do in the case of myoglobin. For example, for a protein molecule consisting of two identical halves which can unfold independently, the value of the heat of denaturation obtained calorimetrically will be exactly twice as large as the value determined using the van't Hoff equation. (This situation is similar to that existing when differences in titration curves between denatured and native molecules are determined by titration and by studying the *p*H dependence of the equilibrium. See Hermans, 1965.) Therefore the calorimetric measurement may in many cases be able to provide information which is otherwise not available.

Finally, it may be pointed out that the use of the calorimeter as a tool in studying macromolecules of biological interest need not be restricted to obtaining information about reversible denaturation of proteins. In fact other uses come easily to mind, but a discussion of these falls outside the scope of this paper.

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