haps be elaborated into an assay more sensitive, but less convenient, than the oxidation by NBS.

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Thermal Denaturation of Myoglobin. I. Kinetic Resolution of Reaction Mechanism*

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ABSTRACT: The thermal denaturation of sperm whale myoglobin was investigated kinetically at pH 6.85 and ionic strength 0.096 in sodium phosphate buffer. Progress curves of the absorbance changes at 290, 409, and 540 mμ were obtained for temperatures between 40 and 90°. Three reaction phases in the denaturation process were resolved experimentally. The first phase was a rapid equilibrium. In the second phase, the reaction rate showed a very sharp dependence on temperature at 80°. This effect was reflected in the Arrhenius plot as a deviation from linearity. The limiting slope of the Arrhenius plot at the higher temperatures gave a value of $\Delta H^{\ddagger} = +109$ kcal/mole. Assuming $\Delta F^{\ddagger} = +30$ kcal/mole, the value $\Delta S^{\ddagger} = +224$ eu was calculated. The third phase was the formation of precipitate. An interpretation of mechanism is suggested, involving a conformational disturbance in the region of the heme group in the first phase, unfolding of the helical regions in the molecule in the second phase, followed by a sequence of polymerization steps, leading to precipitation in the third phase.

The concepts relating to the denaturation of proteins have, in the last decades, been subject to considerable refinement. In view of what is known today about the structure of globular proteins, it is of interest to ask the question: Does the denaturation process take place in a single transition or by means of a series of discrete kinetic steps, which are experimentally demonstrable?

A globular protein, such as myoglobin, consists of a number of helical and nonhelical regions, which are far from being identical in composition and which differ in their interactions with the rest of the molecule. It might be expected that the stability of these regions, for example, toward heat, will not be the same, that is to say different regions of the molecule undergo thermal transitions in different ways at different rates. On the other hand, considering the complexity of a molecule like myoglobin, it is conceivable that a strong cooperative effect exists in the molecule which stabilizes the native configuration and that any single disturbance of this configuration leads to a total loss of the cooperative effect. Such an assumption would be correct, if the denaturation process is a single-stage transition.

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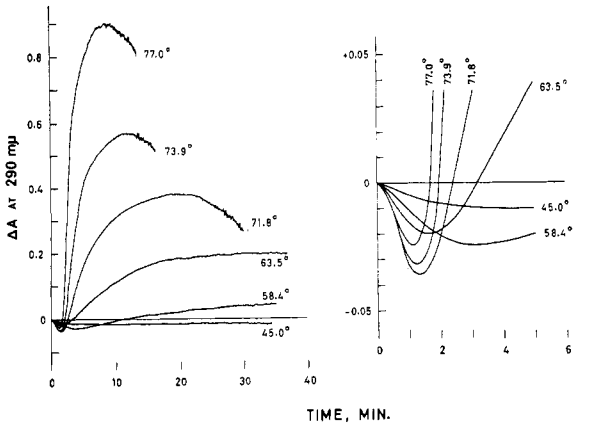

FIGURE 1: Progress curves observed at 290 mμ. Myoglobin concentration was 3.27×10^{-5} M.

The two-state theory of protein denaturation, which treats reversible denaturation as an equilibrium between the native and denatured states, $N \rightleftarrows D$, has been used in the interpretation of denaturation by a number of authors, for example, Neurath *et al.* (1944), Kunitz (1948), Eisenberg and Schwert (1951), Kauzmann (1954, 1959), Schellman (1955), Scheraga (1960, 1962), Tanford (1962), Brandts and Lumry (1963), Brandts (1964), and Acampora and Hermans (1967). The two-state theory has been criticized by Poland and Scheraga (1965) on the grounds that the thermal transitions in globular proteins are much broader than would be predicted by the two-state theory. Poland and Scheraga (1965) have proposed a statistical theory for thermal transitions, which involves the concept that at any particular temperature the state of the protein comprises a distribution of conformational isomers in equilibrium with each other. It is important to note that the two-state theory is a thermodynamic statement, which consequently makes no comment on the kinetic mechanism of the denaturation process.

A number of studies have been reported in the literature describing intermediates in the denaturation of globular proteins. Levy and Warner (1954) and Warner and Levy (1958) showed that in the thermal denaturation of bovine plasma albumin, intermediate polymeric species are formed as a result of sulfhydryl group reactions. Likewise the aggregation of bovine serum albumin in acid solutions was interpreted by Bro *et al.* (1958) in terms of intermolecular thiol–disulfide exchange. Steinrauf and Dandliker (1958) separated a dimer intermediate in the thermal denaturation of bovine serum albumin by means of solubility in 83% acetic acid. The dimer was formed in the first few minutes of heat exposure through intermolecular sulfhydryl–disulfide interchange. The thermal denaturation of bovine β-lactoglobulin was investigated kinetically by Stauff and Ühlein[1] (1955) using 90° light scattering. The progress curves of the denaturation reaction revealed four reaction phases. The first phase, quite remarkably, was manifested in a decrease in the light scattering, the second phase showed a return to the original scattering value, and the later phases indicated polymerization. Scott and Scheraga (1963) described the kinetics of denaturation of ribonuclease using a pH-jump technique (pH 7–0.9) in which the reaction was followed spectrophotometrically at 235 and 287 mμ. The kinetics were interpreted in terms of a two-step reversible mechanism and the possibility was suggested that different sections of the ribonuclease molecule undergo denaturation independently.

The thermal denaturation of myoglobin has not been investigated until recently. Acampora and Hermans (1967) have made equilibrium studies of the thermal transitions of sperm whale myoglobin in the pH ranges of 4–5.5 and 9–13 at temperatures in the range of 20–100°. In the range of pH 5.5–9, precipitation occurred precluding thermodynamic investigation. In a companion paper, Hermans and Acampora (1967) have given a thermodynamic analysis leading to the calculation of $\Delta H_{den}°$ and $\Delta C_p°$ values for the thermal denaturation of myoglobin.

A kinetic study of the thermal transitions of myoglobin was initiated in this laboratory with the aim of elucidating the reaction path of the denaturation process and its dependence on temperature, pH, and solvent. The present work deals with the kinetics of the thermal transitions of sperm whale myoglobin in phosphate buffer (pH 6.85) at temperatures in the range of 25–90°, keeping the pH and ionic strength constant. At least three reaction phases have been separated experimentally. It is suggested that in the first and second phases the kinetic intermediates are monomeric myoglobin species.

Experimental Section

Sperm whale myoglobin was obtained from Seravac Laboratories (Maidenhead, Berkshire). This preparation was at least 95% pure (Awad and Badro, 1967).

Buffer Solution. The solvent used throughout this work was sodium phosphate buffer, 0.050 M in phosphate (pH 6.85), ionic strength 0.096. The pH was measured at 25.0° with a Radiometer pH meter 4 (type RH M4C) with an accuracy of ±0.002 pH unit. Considering that the heat of the second ionization of phosphoric acid is small, $pK_2 = 7.198$ at 25° and $\Delta H° = 987$ cal/mole (Edsall and Wyman, 1958), it was assumed that the pH of the solvent at the higher temperatures was not significantly different from that measured at 25° for the purposes of the present study. A simple calculation shows that a change of temperature from 25 to 90° would result in a pH change of 0.11 unit.

Kinetic Measurements. A freshly prepared solution of ferrimyoglobin was placed in a jacketed cuvet (Scientific Glass Apparatus Co., Inc., Bloomfield, N. J.) in the cell compartment of a Hitachi Perkin-Elmer Model 139 spectrophotometer. The cell was allowed to reach temperature equilibrium at 25.0° by circulating water from a constant-temperature bath. At zero time, the water circulating in the cuvet jacket was replaced with water at the desired temperature from a second constant-temperature bath by turning two stopcocks in the circulation system. The progress curve of the denaturation re-

[1] Cited by M. Joly in 1965, p 81.

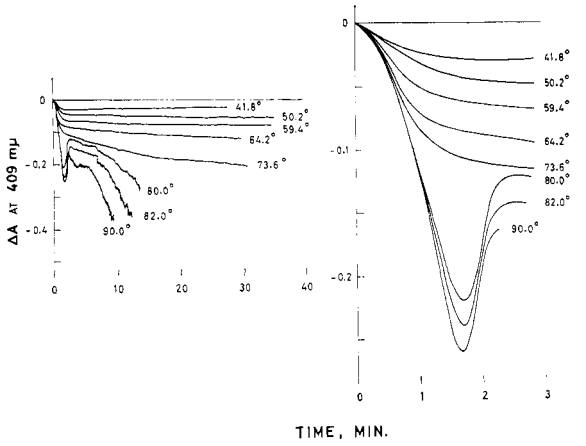

FIGURE 2: Progress curves observed at 409 mμ. Myoglobin concentration was 6.54×10^{-6} M.

action was displayed as an absorbance change on a Beckman log-scale recorder. The temperature of the reaction was measured at the end of the experiment with a mercury thermometer accurate to ±0.05°.

Temperature Response. The rate of temperature rise in the cuvet was determined by means of a phenolphthalein chemical thermometer in a separate experiment. This method takes advantage of the changes in absorbance at 550 mμ, which occur in a phenolphthalein solution, buffered with 0.1 M glycine (pH 9.68), when the temperature is changed (E. S. Awad, N. K. Abed, and F. H. Jumayan, to be published). The method is sensitive to 0.2°. The temperature change in the cuvet, for a number of final temperatures in the range of 40–90° had consistently a rise time (90% of the total temperature change) of 1.5 min or faster.

Results

Preliminary experiments showed that when myoglobin in phosphate buffer at pH 7 is exposed to temperatures up to about 40° and then cooled to 25° within a few minutes, the changes in absorbance were completely reversible even when the duration of the exposure to heat was as long as several hours. At temperatures above 40°, there occurred significant permanent alterations in the spectrum and eventually precipitation. Repetitive scanning of the spectrum during exposure to heat in a Perkin-Elmer Model 202 spectrophotometer at 8-min intervals showed progressive changes in the spectrum over the range of 240–600 mμ, which on a proportionate basis were not the same at different wavelengths. In most regions of the spectrum these changes constituted considerable increases in absorbance. The notable exception was the Soret region around 409 mμ, where decreases in absorbance were observed.

A kinetic study of the thermal transitions of myoglobin at selected wavelengths seemed, therefore, to be an approach that would give fruitful results. The wavelengths chosen were the 290-mμ tryptophan band, the 409-mμ Soret band, and the 540-mμ heme–ligand band. Kinetic runs were performed at a number of temperatures between 40 and 90°. The myoglobin concentrations ranged between 3.27×10^{-6} and 3.27×10^{-5} M. The equilibrium between ferrimyoglobin hydrate (acidic ferrimyoglobin) and ferrimyoglobin hydroxide (basic ferrimyoglobin) is governed by $pK_a = 8.96$ at 25° and $\Delta H° = +6.4$ kcal/mole (Dr. G. I. H. Hanania, personal communication). Thus the myoglobin in our system was entirely in the ferrimyoglobin hydrate form.

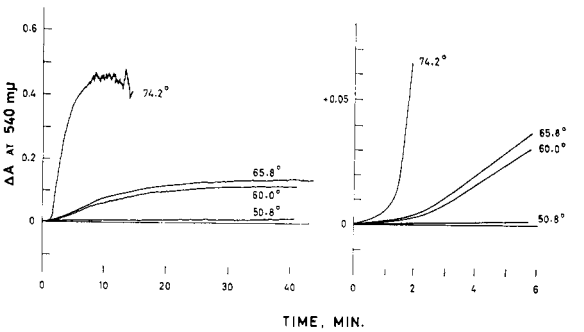

FIGURE 3: Progress curves observed at 540 mμ. Myoglobin concentration was 3.27×10^{-5} M.

Figures 1–3 show, on the left-hand side, tracings of the original recordings of changes in absorbance obtained in representative sets of experiments. On the right-hand side, the initial stages of the observed curves have been replotted, using expanded absorbance and time scales, in order to show more clearly the form of the progress curves. The following features may be noted.

290 mμ. There is an initial rapid decrease in the absorbance, although the maximum change is rather small. This is followed by a second reaction phase in which the absorbance rises to a large positive ΔA value. Finally, the absorbance tends to decrease with considerable noise appearing on the trace. This marks the onset of precipitation. The progress curves at 290 mμ indicate, therefore, at least three distinct reaction phases.

409 mμ. At the higher temperatures (above 80° in Figure 2), the initial rapid and large decrease in absorbance is followed by a rapid and large increase in absorbance. Soon after, precipitation begins. The final absorbance remains less than the absorbance at zero time. At the lower temperatures (below 73.6° in Figure 2), the reversal in ΔA is not seen, indicating that the rate in the second phase is very small at these temperatures. This implies that the reaction rate of the second phase has a very strong and abrupt temperature dependence between 73 and 80°. The plateau regions of the progress curves at temperatures below 73.6° represent asymptotic values of ΔA pertaining to the first reaction phase. Since these values are temperature dependent, it is likely that the first reaction phase is a reversible kinetic step. For, if this step were not reversible, then the same asymptotic value for ΔA should have been observed at different temperatures. The existence of at least three distinct reaction phases is substantiated by the progress curves at 409 mμ, quite strikingly so at temperatures above 80°.

540 mμ. The initial phase shows a very slow and small increase in absorbance, in contrast to the large and rapid increase observed in the second phase. Then follows precipitation. Again three reaction phases are distinguishable.

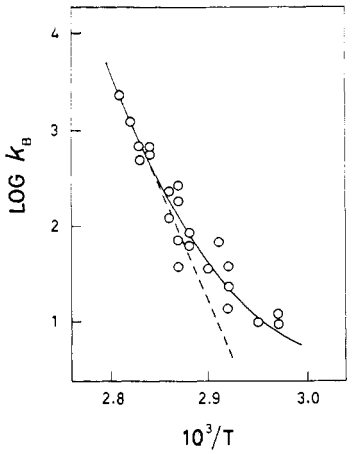

FIGURE 4: Arrhenius plot showing the dependence on temperature of the second-phase reaction rate, k_B, obtained at 290 mμ. Myoglobin concentrations ranged between 3.27×10^{-6} and 3.27×10^{-5} M.

The diversity of the forms of the progress curves at 290, 409, and 540 mμ is due to the fact that different electronic transitions within the myoglobin molecule are being observed at these wavelengths. The reaction scheme of least complexity, which is needed for a kinetic description of the progress curves at 290, 409, and 540 mμ, is of the type

$$N \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} I_A \xrightarrow{k_2} I_B \xrightarrow{k_3} P$$

where N represents native myoglobin, I_A and I_B intermediate species, P precipitate, and k_1, k_{-1}, k_2, and k_3 are kinetic constants.

The initial rates, k_B, relating to the second reaction phase were determined from the original traces of the progress curves at 290 mμ. The parameter, k_B, is defined by the equation

$$k_B = \frac{1}{c_0} \lim_{t \to 0} \frac{d\Delta A}{dt}$$

where c_0 is the original myoglobin concentration. An Arrhenius plot for the values of k_B is given in Figure 4, showing a deviation from linearity at the lower temperatures. The limiting slope of the Arrhenius plot at the higher temperatures yields for the enthalpy of activation a value of $\Delta H^{\pm} = +109$ kcal/mole, using the equation $d(\ln k_B)/d(1/T) = -\Delta H^{\pm}/R$.

Discussion

Although k_2 and k_B relate to the same reaction step, these parameters are not identical. While k_2 is a first-order constant, k_B is a rate which has been normalized with respect to the initial myoglobin concentration. The units of k_B are: absorbance $\times$ M^{-1} sec^{-1} = M^{-2} cm^{-1} sec^{-1}. The first-order constant, k_2, may be defined by the relation

$$k_2 = \frac{1}{\Delta A_{max}} \lim_{t \to 0} \frac{d\Delta A}{dt}$$

where ΔA_{max} is the asymptotic value of ΔA as $t \to \infty$, for that reaction step. Thus $k_B c_0 = k_2 \Delta A_{max} = k_2 c_0 \Delta \epsilon_{max}$, where $\Delta \epsilon_{max}$ is the difference in the molar absorbancies of the intermediate species I_B and I_A. Hence $k_B = k_2 \Delta \epsilon_{max}$.

The value for ΔA_{max} could not be obtained with certainty from our data. So the value of $\Delta \epsilon_{max}$ is not accessible and k_2 cannot be calculated from the relation $k_B = k_2 \Delta \epsilon_{max}$. However, since $\Delta H^{\pm}$ is determined from the slope of a log plot, it follows that the value we have obtained is an authentic Arrhenius energy. The deviation from linearity in the Arrhenius plot at the lower temperatures is at least in part due to the rapid equilibrium preceding the second reaction step. It can be readily shown that for the reaction sequence we have assumed, in which the first step is a rapid equilibrium, the apparent rate constant for the second step is given by $k_{app} = k_2 K_1/(1 + K_1)$, where $K_1 = k_1/k_{-1}$ is the equilibrium constant for the first step. At the higher temperatures, $K_1 \gg 1$, and hence $k_{app} \approx k_2$. Thus our $\Delta H^{\pm}$ value is correctly related to k_2.

According to Joly (1965), the order of magnitude of $\Delta F^{\pm}$ is 22 $\pm$ 5 kcal/mole for a large number of known cases of protein denaturation. For the thermal denaturation of hemoglobin at 60° (pH 6.76), the values range between 25.5 and 28.1 kcal/mole depending on the ionic strength (Joly, 1965; Eyring and Stearn, 1939; Lumry and Eyring, 1954). If it is permissible to assume a value of $\Delta F^{\pm} = +30$ kcal/mole for myoglobin relating to our k_B at 80° and pH 6.85, then the entropy of activation, $\Delta S^{\pm} = +224$ eu, is obtained from the relation $\Delta S^{\pm} = (\Delta H^{\pm} - \Delta F^{\pm})/T$.

The kinetics of the first reaction phase, $N \rightleftarrows I_A$, could not be studied, because the progress curves in the first phase were barely lagging behind the temperature response in the cuvet; but the fact that a change in absorbance was observed, shows the existence of a kinetic step. The formation of precipitate in our experiments did not affect the accuracy of the measurement of k_B from the progress curves at 290 mμ, because these k_B values were obtained from regions of the progress curves which were sufficiently well removed from the precipitation phase. The progress curves we obtained at 409 and 540 mμ were not adequate for obtaining values of k_B.

Reaction Mechanism. The absorbance change at 409 mμ in the first reaction phase is large, whereas the corresponding changes in absorbance at 290 and 540 mμ are quite small (Figures 1–3). This suggests that in the initial phase there occurs some disturbance in the region of the heme group, which may be due to a minor local alteration in the tertiary structure, which in turn gives rise to changes in the interactions of the heme group with the amino acid side chains in the vicinity. During this phase, the absorbance changes at 290 mμ indicate that the tryptophan (also tyrosine and phenylalanine) environment remains essentially unaltered. The lack of response at 540 mμ suggests that no change has occurred in the heme–ligand binding. The large changes in absorbance which take place in the second reaction phase at 290, 409, and 540 mμ may be ascribed to extensive alterations in the secondary and tertiary structures of the molecule. It is necessary to postulate a phase of

polymerization prior to the appearance of precipitate, because at pH 6.85 there would be a sufficient number of ionic groups to hold the monomer in solution. The following reaction scheme for the thermal denaturation of myoglobin may be formulated.

$$N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} I_A \xrightarrow{k_2} I_B \xrightarrow{k_2'} (I_B)_2 \xrightarrow{k_2''} \cdots \xrightarrow{k_2^{(n-1)}} (I_B)_n \xrightarrow{k_3} P$$

Myoglobin does not contain sulfhydryl or disulfide groups (Edmundson, 1965). In other proteins, these groups are active in the thermal denaturation process by forming intermolecular disulfide linkages in the polymerization phase. In myoglobin, intermolecular hydrophobic bonding must be a major driving force in the polymerization phase.

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