Kinetics of Conformation Change of Sperm-Whale Myoglobin. I. Folding and Unfolding of Metmyoglobin following pH Jump†

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ABSTRACT: Using a stopped-flow spectrophotometer we have followed the denaturation of sperm-whale myoglobin following pH changes in the range 3–4.5. From an analysis of the time course of the ultraviolet (uv) absorption in the range from 350 to 420 nm, it is concluded that in the transition range a single step is present, while at pH below 3.8 the reaction takes place in two steps. The unfolding rates range from 10^{-2} sec^{-1} at pH 4.4 to 10^{3} and 10 sec^{-1} at pH 3. The presence of a slow step following the fast step(s) is evident from the observation that the time course of refolding depends markedly on the time interval during which the protein is let to denature, long past the moment when no further changes in the spectrum of the denatured protein are observed. Unfolding conditions were established under which only the first (= the observable) step has taken place when the refolding experiment is initiated. Doing this, a single rate for refolding is observed in the transition range, which, at the midpoint of the transition, is equal to the rate of unfolding (both 10^{-2} sec^{-1} at pH 4.4). At higher pH, two steps are observed, and the rates for these two steps become independent of pH, being *ca.* 0.2 and 0.08 sec^{-1} between pH 5 and 6. These results are discussed in terms of a scheme of four species, native (N), intermediate (X), denatured (D), and modified denatured (D*), which are converted one to the other in that order. Form X is not observed in the transition range, and the experiments on refolding after short denaturation times regard the reaction from D to N. The observation of a single step in the transition range and of the equality of the unfolding and refolding rates at the midpoint of the transition are shown to be a reflection of the two-state nature of the transition, demonstrated before.

The problem of the denaturation of proteins is important as a source of information leading to a better understanding of protein structure in solution, and a considerable number of investigations on various proteins have been documented in recent years (Tanford, 1968, 1970, and references therein). The kinetics of acid denaturation and refolding of hemoglobin have been studied exhaustively, and this work has resulted in a series of papers starting with the work of Steinhardt and Zaiser (1953) and continuing to this date (McGrath and Steinhardt, 1971). The very number of these articles testifies to the complexity of the unfolding reaction of hemoglobin. Schechter *et al.* (1970, 1971) have studied the kinetics of refolding of staphylococcal nuclease. Their data are, so far, incomplete in that a study of the denaturation equilibrium and of the kinetics of unfolding has not yet been published. Still, the results obtained with this enzyme appear much more straightforward than those obtained with hemoglobin. Results obtained with ribonuclease by Scott and Scheraga (1964) are incomplete in that no refolding kinetics were reported. In the present series of three articles we present a study of the kinetics of unfolding and refolding of myoglobin, and at the end of this series a relatively complex mechanism of this process is postulated.

The three-dimensional structure of myoglobin has been completely determined (Kendrew *et al.*, 1969). Myoglobin is a globular protein with 75% of helicity (Urnes, 1968), and its monomeric behavior in solution, the absence of disulfide bridges, as well as the Soret absorption band which results from the interaction of heme with globin, provide great advantages in an investigation such as ours. These are, of course, nearly the same advantages which were exploited by Steinhardt and coworkers in their work on hemoglobin. As it turns out, we shall see that heme–globin interactions dominate the kinetics of the conformation change; hence information of the structural relationship between globin and its prosthetic group can be expected, rather than information about the kinetics of the conformation changes of globin which might be generalized to describe the behavior of proteins in general. Thus, much of the complexity of the final description of these kinetics has its origin in properties of the heme, and are thereby unique to myoglobin and hemoglobin. In hemoglobin, the situation is further complicated by the fact that the protein is made up of four polypeptide chains, of two different types.

Kinetics of myoglobin denaturation were previously studied by several authors. Litman (1966) studied the denaturation by guanidine hydrochloride, Khalifah (1968) that by urea, Awad and Deranleau (1969) studied the time course of thermal denaturation at pH 6.85, and most recently Marks *et al.* (1971) report the kinetics of denaturation of myoglobin by cupric ion. In all these studies, the greatest difficulties were encountered in explaining measurements of the reversal of the unfolding. Generally, the presence of heme–protein aggregates was held responsible for these difficulties. This conclusion is in agreement with the results of our study reported here. Furthermore, we indicate conditions whereby these difficulties can be avoided.

In previous papers (Acampora and Hermans, 1967; Hermans *et al.*, 1969), the effect of temperature, pH, and some other parameters on the equilibrium system of the reversible denaturation of sperm-whale myoglobin has been investigated extensively. A reversible transition from native structure to denatured structure is observed at pH from 4.8 to

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4.2 as indicated by the parallel change of both optical density in the Soret band and optical rotation at 233 nm. This transition is also temperature dependent but in a somewhat complicated way, in that at room temperature the stability is maximal. We now investigate the kinetics of the structural transition of metmyoglobin at 25° within this region, as well as at lower pH down to pH 2.5, and, using the results of these unfolding experiments, investigate the refolding kinetics. Subsequent papers in this series will deal with the kinetics of unfolding and refolding of carbon monoxide myoglobin in the presence of dissolved oxygen, which will provide additional information about the details of the process, and with the kinetic behavior of apomyoglobin and related experiments, which all together will lead us to postulate a final mechanism.

Experimental Section

Material and Reagents

Sperm-whale myoglobin was purchased from Seravac Laboratories. Other chemicals were all reagent grade material.

Methods

Preparation of Solutions. The concentration of myoglobin solution was determined using either the optical density at 423 nm, of a suitable dilution in a buffer containing potassium cyanide, and a molar extinction coefficient of 109,000 M^{-1} cm^{-1} determined by Hanania *et al.* (1966) or the optical density at 409 nm of a suitable dilution and a molar extinction coefficient of 157,000 M^{-1} cm^{-1}. The concentrations calculated by using these two methods are identical within experimental error.

Citrate–KCl buffers of ionic strengh 0.2 (0.05 M in citrate) were used in the pH range from 2.9 to 5.0. For the pH range of 5.0–6.5, a citrate concentration of 0.03 M was used. Some buffers with lower buffer strength (0.003 M in citrate concentration) were used for refolding experiments, but the ionic strength was always kept constant (0.2) throughout this investigation.

Measurements. pH measurements were carried out with an Orion Model 801 digital pH meter, using a Fisher No. 13-639-1 glass electrode and a Radiometer Type K-130 Calomel electrode, standardized according to Bates (1964).

The optical density measurements at fixed wavelength were performed on the Zeiss PMQ II spectrophotometer with thermostated cell holder. The ultraviolet and visible absorption spectrum measurements were carried out on the Cary Model 14 recording spectrophotometer.

Optical rotatory dispersion measurements or single wavelength measurements were made using the Cary 60 spectropolarimeter using fused quartz cells of 10-mm path length.

For slower reactions, the kinetic measurement was performed spectrophotometrically on the Cary Model 14 recording spectrophotometer. By using a simple two syringe hand-mixer, the mixing of equal volumes of myoglobin working solution and acidic buffer may be completed in 1 sec and a first reading can usually be made 4 sec after the actual mixing was done. Kinetic measurements followed by optical rotation were performed with this method.

For fast reactions, the kinetic measurement was done using a Durrum-Gibson stopped-flow spectrophotometer. Under optimum conditions, a mixing dead time of 3 msec and a value of signal-to-noise ratio equal to 1000 were obtained (100-mV total signal, 100 msec/cm at 409 nm, photomultiplier voltage at 350 V, slit width 1.0 cm). The temperature was controlled to within ±0.1°.

The refolding experiments required a double-mixing setup. the first mixing (for the unfolding process) was performed with the hand mixer. The outlet tubing of the hand mixer was directly connected to one of the drive syringes on the stopped-flow apparatus through the reservoir syringe connector. The denatured protein solution can fill the drive syringe in 1 sec, and the stopped-flow apparatus will be ready for the second mixing (to obtain refolding) in approximately 10 sec.

Results

In order to determine the time dependence of the unfolding, we follow the absorption changes in the Soret band (*ca.* 410 nm) and the optical rotation near 233 nm, which are known to be sensitive measures of unfolding equilibria (Harrison and Blout, 1965; Breslow *et al.*, 1965; Acampora and Hermans, 1967).

Time Dependence of the Spectra during Unfolding. The kinetic traces were first obtained at a number of different wavelengths between 350 and 420 nm. The time dependence of the spectra (shown in Figure 1) was constructed from these kinetic traces obtained when myoglobin was denatured at different pH between 3.09 and 4.25. The spectral shift as seen in these diagrams may be used to reveal individual steps composing the entire reaction. This method has been used by Polet and Steinhardt (1969) in their studies of the acid denaturation of hemoglobin, a protein which is structurally similar to myoglobin and which in many respects behaves as does myoglobin.

As the example in Figure 1F, the unfolding in the transition range ($f_N \sim 0.5$ at equilibrium) is primarily a one-step process. After the initial small red shift of the peak by about 2 nm, which we do not count as a step related to conformation change, there follows the major step of the reaction as indicated by the successive decrease of the peak, a single isosbestic point at 383 nm being maintained throughout the entire reaction. At lower pH, the spectral change is more complex as we can see Figure 1A–E, and can be separated into three stages. First is a rapid and small red shift of the peak to 411 nm, then the left side of the peak moves successively to longer wavelengths until an isosbestic point is obtained. At the same time, the peak at 411 nm decreases to give approximately half of the total change. In the second step, the peak at 411 nm continues to decrease; there is an isosbestic point and one observes the ascending of a broad peak at 370 nm. We consider these to be the major steps in the change of protein conformation; they are followed by the third step, in which the spectrum leaves the isosbestic point and completes the new broad peak at 370 nm; this step accounts for the last 10% of the overall reaction as estimated by the absorption change at 411 nm.

Compared with results on the unfolding of hemoglobin (Polet and Steinhardt, 1969; Allis and Steinhardt, 1969), we note that the rates are much larger. Thus, we were unable to perform experiments below pH 3 while with hemoglobin the reaction could be studied at pH 2, where the spectra decay in a more complicated fashion than noted here for myoglobin.

Kinetic Plots of the Unfolding Process.[1] The rate of the reaction was analyzed by the first-order plot method in which $\log (A - A_\infty)$ is plotted versus time, t. The kinetics are com-

[1] For illustrations reporting these results, the reader is referred to Shen's dissertation (1971).

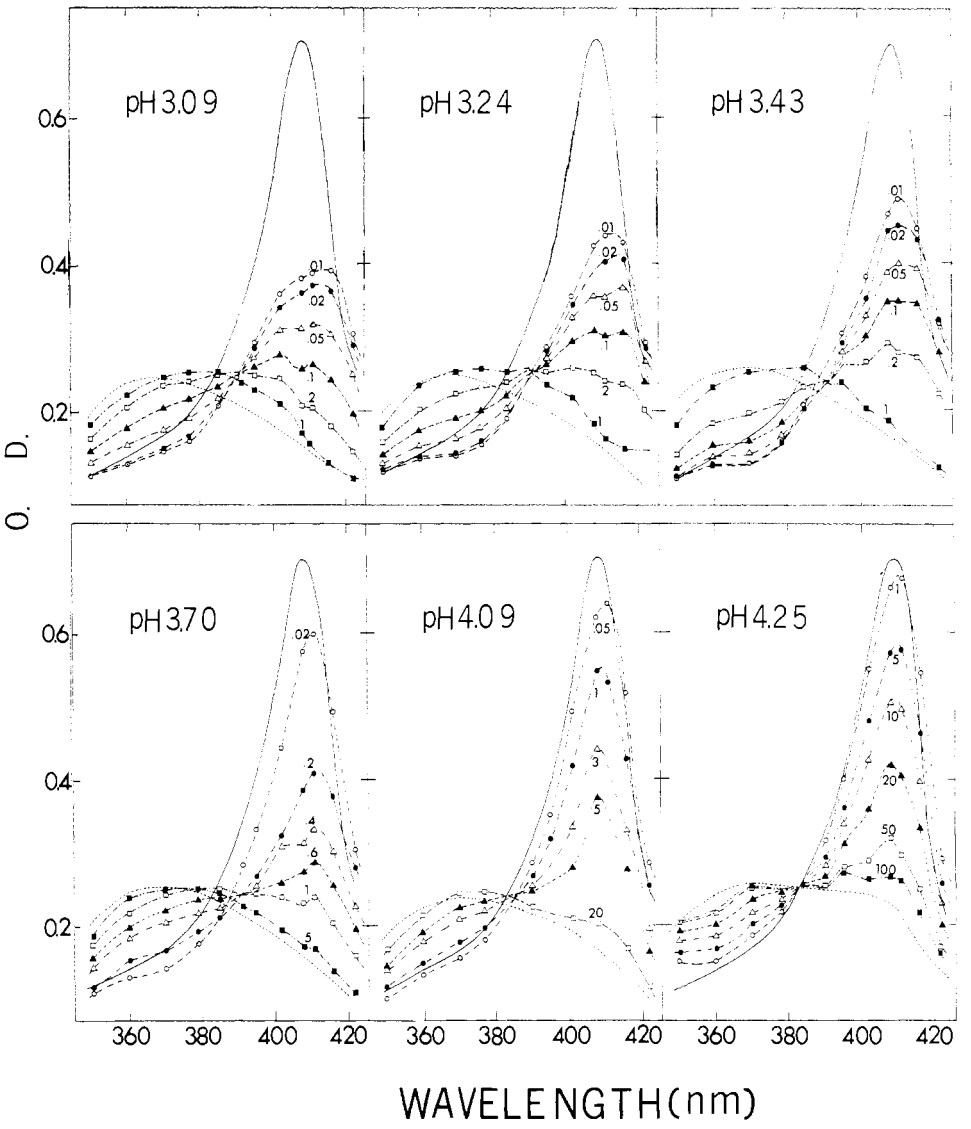


FIGURE 1: The time dependence of the spectra during the unfolding process of metmyoglobin following pH jump, at pH (A) 3.09, (B) 3.24, (C) 3.43, (D) 3.70, (E) 4.09, and (F) 4.25. The solid lines and dotted lines without points are measured on a Cary Model 14 spectrophotometer; the former is the spectrum of native myoglobin at pH 6.5, the latter that of a solution having been at low pH for *ca.* 10 min. The other curves were constructed from the time dependence of the absorption at the indicated wavelengths (time in seconds).

plex over most of the pH range, the first-order-type plots give a curve which corresponds to a three-step process at low pH and an apparent two-step process in transition range.

The change between the two kinetic patterns is continuous, and we cannot say if one of the steps gradually becomes undetectable because the change in absorption corresponding to the steps becomes zero, or because the difference in slope for two steps becomes smaller, when the pH approaches the transition pH. We designate the *apparent* first-order rate constants for these three steps as λ_1, λ_2, and λ_3, calculated from the slope of the three line segments in each plot. At low pH, λ_1 and λ_2 show considerable difference, while λ_2 and λ_3 are almost the same. As the pH of the denaturation is raised, the difference in λ_1 and λ_2 is reduced.

It is of interest to compare these conclusions regarding the different steps with those reached on the basis of inspecting the overall spectral changes in this process. In the transition range the unfolding process was concluded to be a single step from the spectra (Figure 1F), but another step can be separated in the kinetic plot, while at low pH we find three steps with both methods. At lower pH, the kinetic plots indicate that the first stage of the unfolding reaction occupies the first half of the entire reaction. The second stage ends when 78% of the total reaction is completed. The duration of these three stages coincides with what we deduced from the overall spectral change.

As a consequence, we feel justified in interpreting the results on the basis of a series of consecutive or parallel reactions between a small number of reasonably well-characterized states of the molecule; starting product, end product, and one or more transiently stable intermediates.

Renaturation Studies. Dependence on Time Elapsed at Low pH and on pH of Renaturation. The spectral change which accompanies acid denautration of myoglobin was found to be generally reversible by again raising the pH of the solution. Under more careful investigation, we found that the extent of reversibility and the velocity of the renaturation was very strongly dependent on the duration of the preceding unfolding, which we shall call Δt. Figure 2 shows the extent of the renaturation in terms of the Soret band absorp-

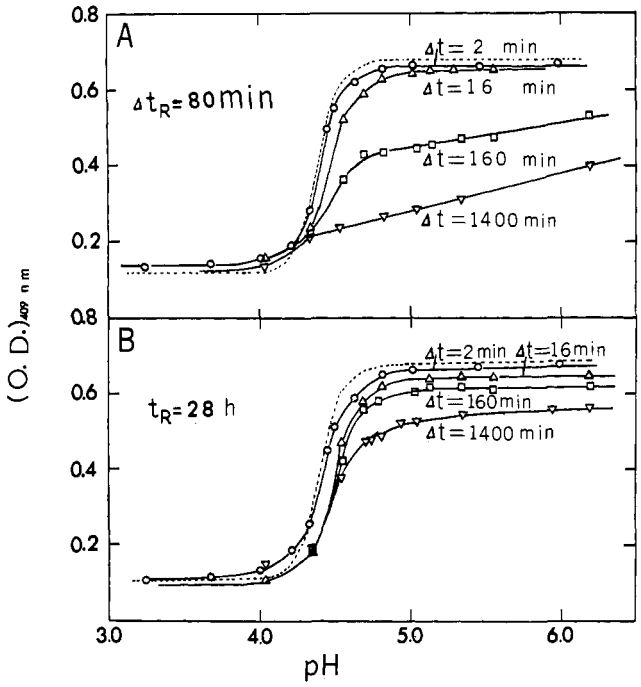


FIGURE 2: Transition curves for the refolding of metmyoglobin which has been unfolded at pH 3.4 with different denaturation time (Δt). The curves are measured after (A) 80 min and (B) 28 hr of refolding. The transition curve of metmyoglobin which has not been unfolded has also been indicated (dashed).

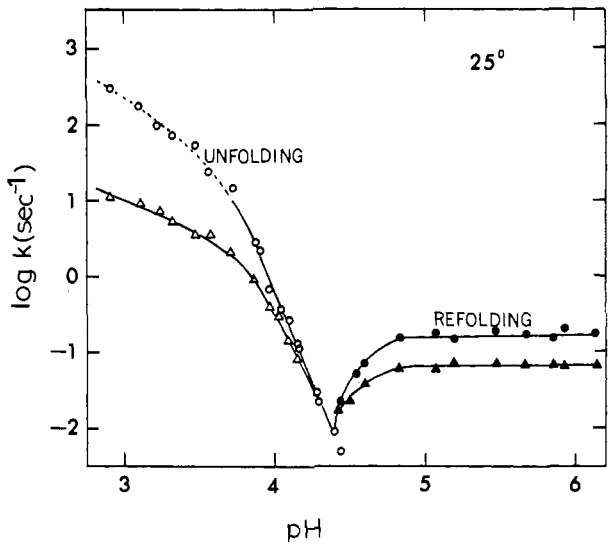


FIGURE 3: The pH dependence of the logarithm of the apparent rate constants for unfolding and refolding. The dashed-line segment indicates that these points are minimum values consistent with the experimental data. (○) Rate constants for unfolding, step 1; (△) rate constants for unfolding, step 2; (●) rate constants for folding, step 1; (▲) rate constants for folding, step 2.

tion as a function of renaturation pH after the protein was denatured with different Δt values at pH 3.4. After 80 min of renaturation, the curve with Δt equal to 2 min almost came back to the position obtained on denaturing a protein which has never been denatured, while those with Δt equal to 160 and 1400 min are still far from that position. After 28-hr renaturation, those curves approach the normal position more closely, although the midpoints do not appear to converge to the midpoint for the forward transition and for the reverse transition with Δt = 2 min.

Thus, it appears possible to define two different acid transitions of myoglobin, one of which may be studied in relatively short times, and one (at a higher pH) which is established (and reversed) considerably more slowly. In earlier work (Acampora and Hermans, 1967) the first transition was studied. The kinetic results presented in this paper justify the choice of an equilibration time of 1–2 min used in the earlier work, which had been chosen more empirically. We shall continue to designate the transition studied earlier as the denaturation equilibrium. Reasons for this will follow from results reported in this and the two following papers.

As we have seen, the denaturation of myoglobin at pH 3.4 has a half-time approximately equal to 0.1 sec, and subsequent reactions do not give any further appreciable change in optical density after approximately 1 min. Hence, we conclude that there must exist a subsequent and extremely slow reaction, which is not detectable spectrophotometrically, following the major unfolding reaction. In the transition range, this reaction does become detectable as a second step (see above), not by virtue of a difference in absorption spectrum between the first and subsequent denatured species, but because the concentration of native material, which is here in measurable equilibrium with the first denatured species, slowly decreases as the denatured material reacts to form the later species.

Reversibility. The reversibility of the denaturation reaction was defined not only by the return of the uv absorption to the original value, but also by showing that the denaturation kinetics of material which has been denatured for a short time, then renatured, were within experimental error the same as for the native material. It was also found that the denaturation kinetics of incompletely renatured material (obtained with insufficient renaturation times following long exposure to denaturing conditions) were the same as those of native material. This supports the view that the regain of optical density is due to the re-formation of intact myoglobin molecules, and not of partly folded species.

Kinetics of Refolding. We are interested in finding conditions under which we can observe the reversal of the initial major steps of the unfolding process. The formation of slowly reversed denatured products at long times will prevent this, since the slowest step will be rate limiting in reversing the sequence of steps, and the faster steps will be unobservable. (This is so whenever there is at least one transient stable intermediate, and the rate for the step leading to the intermediate is much larger than that leading from it.) By decreasing the time elapsed under denaturing conditions (Δt), one may hope to obtain denatured products which will refold more rapidly. This is perhaps not always the case, but will hold if there are no unexpected differences in the nature (and hence in the relative stabilities) of the various species which are detected under denaturing conditions and those which are detected under the conditions favoring refolding.

We have chosen a pH of 4.16, *i.e.*, as close to the transition as possible while still giving essentially complete unfolding, as the standard unfolding conditions. By following the unfolding at this pH at 409 nm we found that with Δt equal to 12 sec we shall have considerable reaction by the first step, and a very limited amount of reaction by the second step.

The kinetics of renaturation were analyzed with first-order kinetic plots which show that this reaction is a two-step process throughout the entire pH region of interest. The apparent first-order constants of these two steps designated as λ_{-1} and

λ_{-2}, are distinct at higher refolding pH and approach the same value when the refolding pH is close to the transition pH, as in the case of unfolding kinetics. The constants λ_{-1} and λ_{-2} are independent of the pH at which the protein is refolded above the transition region. The refolding rate does not attain the high values typical of the unfolding reaction at low pH.

The apparent first-order rate constants for both unfolding and refolding are plotted in Figure 3 as a function of pH; a tree-shaped diagram is obtained. A most gratifying finding from this diagram is that all four parameters (λ_1, λ_2, λ_{-1}, λ_{-2}) meet at the same single value at the midpoint of the transition region.

Similar experiments with hemoglobin by Steinhardt *et al.* (1958) give very different results. One reason for the difference may be the difference in time required to obtain the denatured protein, which is 10 sec for myoglobin, at least 30 min for hemoglobin. If reactions subsequent to the first step as found with myoglobin are of similar nature and rate in the two proteins, then the long time needed to denature hemoglobin would ensure that one or more of the subsequent steps occurs during the time required to denature. In that case the rate of the reversal of the unfolding would be determined by completely different processes for the two proteins.

Discussion

As revealed by the shifting of the spectra during the denaturation, the unfolding of metmyoglobin is a multistep process. We demonstrated the existence of an isosbestic point during a significant part of the reaction. This is usually taken as evidence of the existence of only two species. Hence we proposed to limit the number of species and, therefore, the number of steps in the mechanism, to a minimum consistent with the observed changes of the spectrum. The kinetic plots confirm the division into three steps and the approximate duration of the steps, and thereby offer further support for this approach to describing the reaction.

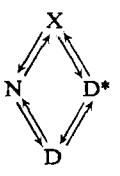
More precisely, we would like to think in terms of a first-order reaction scheme containing four species. Various possible choices are: a sequential scheme with a single route leading to the end product, a parallel scheme where the end product can be reached *via* different sequential paths, and a scheme with transient states not on the reaction paths (these may be considered to be erroneously folded states, Ikai and Tanford, 1971).

The choice between schemes is not so easy to make, since formally each added species simply produces one other relaxation phenomenon in *any* scheme. Since we have evidence for three different relaxation processes, we could propose

$$N \rightleftharpoons X \rightleftharpoons D \rightleftharpoons D^*$$

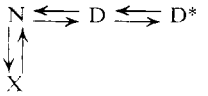
I

according to the sequential scheme (one should not at this moment attach any meaning to the symbols, except in that they specify four distinguishable conformations)



II

according to the parallel scheme, and



III

according to the scheme with abortive intermediates.

All of these schemes are special cases of a general scheme, in which each of the four species N, X, D, and D* can be turned into any other species. A given special scheme is then obtained by setting selected rates to zero. In this light, Scheme III is a special case of II. Ikai and Tanford (1971) use a careful analysis of kinetic data on cytochrome *c* to elect Scheme III. Ikai and Tanford's test is applied in the transition range and requires that two different rate constants determine the behavior. Since we find that λ_1 and λ_2 become equal in the transition range, we cannot apply their test.

With ideal data we might be able to determine all the rate constants in a generalized scheme, but we do not believe this to be practical in our case. Our choice has been to use other experimental evidence not directly connected with the kinetic plots. These results will be discussed below and in the following two articles.

The coincidence of the changes in optical rotation and optical density in denaturation and renaturation experiments at pH's where the reaction is not too rapid to be followed with the spectrophotometer (*cf.* the following article in this series) suggests rather strongly that the form D, which is by definition produced later than form X, is a grossly unfolded species in which the protein–heme contact is lost or grossly modified. (This is the denatured myoglobin molecule which, in equilibrium with the native molecule, has been the subject of thermodynamic analysis; Hermans and Acampora, 1967.) This conclusion is supported by the fact that the forward and reverse rate constants are equal at the midpoint of the equilibrium transition, if the forward reaction is not allowed to proceed beyond the major unfolding step. Also, the conclusion is consistent with the observation that only a single rate is noted in the transition range, although there are two rates outside the transition range, in view of the demonstrated two-state character of the transition equilibrium (Hermans and Acampora, 1967). Only a single step should be observed kinetically for a two-state transition (Tanford, 1968, 1970). This is rigorously true if the observation is made following a small perturbation of the equilibrium. In our case the perturbations are large, but apparently, any pH-dependent changes which occur in the native molecule or in the denatured molecule and which might give rise to another step in our plots, are rapid or unimportant or both.

It is to be concluded that species X does not accumulate at any time when the reaction takes place in the transition range.

The results of the reversibility studies show that the unfolding of metmyoglobin is followed by slow and spectrophotometrically undetectable reactions in which some portion of the protein is irreversibly denatured. The reversal of these reactions becomes rate limiting in a subsequent refolding reaction. The form which is produced we shall call D*. This phenomenon also has been seen with hemoglobin denaturation. Steinhardt *et al.* (1958), in their studies on the refolding kinetics of acid-denatured ferrihemoglobin, found that a small amount of protein is denatured in a competing reaction which yields the irreversibly denatured material, and they referred to this as the "lost protein." As regards the kinetics, they

found that these were neither first order nor second order, with both analyses showing a rapid initial step which counted for approximately 10–20% of the total reaction; anomalies were found in the figure in which refolding rate constants were plotted *vs.* pH and no suitable interpretation could be found. Our results suggest that the anomalies can be avoided by choosing the proper exposure of the protein to the denaturing conditions. Furthermore, the fact that under the conditions of these experiments the folded hemoglobin molecule is at least a dimer, perhaps a tetramer, consisting of two different types of protein chain, will tend to complicate the refolding kinetics.

The kinetic plots indicate the occurrence of a third and slowest relaxation process at low pH. It is possible that at some pH the formation of the hard to renature species D* is accompanied by a spectral change. It is also possible (though unlikely) that a small fraction (~10%) of the protein follows a different and slower route to unfold (*via* another transiently stable intermediate). We have no further information about what happens during this third stage of the low-pH kinetic plots, and do not take it into account in the proposed mechanism.

The appearance of form D*, the form which is more difficult to convert back to the native conformation, can be prevented even with completion of the major conformation change. For this reason, we favor placing form D* in isolation at the end of the scheme, as in I and III. This is also suggested by the constancy of the optical density changes associated with the two steps over the pH range 2.9–3.7, which implies that all the material is first converted to X, then to D and finally to D*.

We shall provide evidence that the nature of species D* and the rates of the transformations are dominated by effects due to the presence of the heme group. However, this is not obvious on the basis of the data so far presented, since two-step analyses have been used to explain kinetic results for several proteins: ribonuclease (Scott and Scheraga, 1963), staphylococcal nuclease (Schechter *et al.*, 1970), and cytochrome *c* (Ikai and Tanford, 1971), to name several. Also, the observed rates (except that for the reversal, D* → D) are neither excessively large nor excessively small. The unfolding rates do rise to very large values at low pH (pH 3), but it should be realized that here the unfolding equilibrium constant $K_{unf} = 10^{12}$ and rising as $[H^+]^6$ or thereabouts (Hermans and Acampora, 1967); it would be surprising if such an enormous value for K_{unf} were *not* associated with an unfolding rate several orders of magnitude higher than the rate observed when $K_{unf} \approx 1$.

References

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

Allis, J. W., and Steinhardt, J. (1969), *Biochemistry 8*, 5075.

Awad, E. S., and Deranleau, D. A. (1968), *Biochemistry 7*, 1791.

Bates, R. G. (1964), Determination of pH, Theory and Practice, New York, N. Y., Wiley.

Breslow, E., Beychok, S., Hardman, K. D., and Gurd, F. R. N. (1965), *J. Biol. Chem. 240*, 304.

Hanania, G. I. H., Yeghiayan, A., and Cameron, B. (1965), *Biochem. J. 98*, 189.

Harrison, S. C., and Blout, E. R. (1965), *J. Biol. Chem. 240*, 299.

Hermans, J., and Acampora, G. (1967), *J. Amer. Chem. Soc. 89*, 1547.

Hermans, J., Puett, D., and Acampora, G. (1969), *Biochemistry 8*, 22.

Ikai, A., and Tanford, C. (1971), *Nature (London) 230*, 100.

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

Khalifah, R. G. (1968), Ph.D. Dissertation, Princeton University.

Litman, B. (1966), Ph.D. Dissertation, University of Virginia.

Marks, R. H. L., Cordes, E. H., and Gurd, F. R. N. (1971), *J. Biol. Chem. 246*, 466.

McGrath, W. P., and Steinhardt, J. (1971), *Biochemistry 10*, 1629.

Polet, H., and Steinhardt, J. (1969), *Biochemistry 8*, 857.

Schechter, A. N., Chen, R. F., and Anfinsen, C. B. (1970), *Science 167*, 886.

Schechter, A. N., Epstein, F. H., Chen, R. F., and Anfinsen, C. B. (1971), *Fed. Proc., 30*, 1288 Abstract.

Scott, R. A., and Scheraga, H. A. (1963), *J. Amer. Chem. Soc. 85*, 3866.

Shen, L. L. (1971), Ph.D. thesis, University of North Carolina, Chapel Hill.

Steinhardt, J., and Zaiser, E. M. (1953), *J. Amer. Chem. Soc. 75*, 1599.

Steinhardt, J., Zaiser, E. M., and Beychok, S. (1958), *J. Amer. Chem. Soc. 80*, 4634.

Tanford, C. (1968), *Advan. Protein Chem. 23*, 121.

Tanford, C. (1970), *Advan. Protein Chem. 24*, 1.

Urnes, R. (1965), *J. Gen. Physiol. 45*, 75.