# Kinetics of Conformation Change of Sperm-Whale Myoglobin. II. Characterization of the Rapidly and Slowly Formed Denatured Species (D and D*)†

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ABSTRACT: This paper describes results which give information about the nature of the acid-denatured form of metmyoglobin observed after a short time (D), and the species occurring at later times (D*), which does not differ appreciably from D in absorption spectrum, but which is much slower to renature when taken to high pH. It is found that the optical rotation at 233 nm changes in parallel with the ultraviolet (uv) absorption when D is produced near the transition range. It is also found that the denaturation of carbon monoxide myoglobin in the presence of oxygen, and subsequent renaturation, yields metmyoglobin in the proportion to which the denaturation had progressed. It is concluded that species D may be designated as the reversibly acid-denatured metmyoglobin molecule. Refolding from species D* was investigated using both uv absorption and optical rotation as a measure. The helicity (as indicated by the latter) is found to increase considerably more rapidly than the amount of renatured protein as indicated by the former. Species D* produced at pH 4 is found to be excluded from the gel in chromatography on Sephadex G-200. At pH 3, species D* chromatographs as two components, the high molecular weight fraction containing heme and protein in a molar ratio of about 2:1, the low molecular weight fraction consisting of probably mostly monomeric protein free of heme. It is concluded that when renaturing D*, the globin chains tend to form the native, largely helical structure to a great extent. Complete regain of helicity and *any* regain of the absorption is accomplished more slowly, *via* dissociation of molecules of heme and protein from the aggregates to form myoglobin molecules.

In the preceding article, we have presented the kinetics of unfolding and refolding of sperm-whale metmyoglobin. We concluded that the unfolding kinetics can be accounted for with the presence of four species: N, X, D, and D*, which appear in that order, while the refolding (of molecules which had been unfolded to conformation D) involves three species: D, X, and N. (The intermediates in the two reactions need not be the same species.) We now report somewhat unrelated experiments which tell us something about the structure of species D and D*. (A) Species D is short-lived, but is observable for a few minutes. Also, at pH not too far below the transition pH, the formation of D is reasonably slow. This permitted us to study the time dependence of the optical rotation of the solution as D was being formed. Optical rotation changes indicate changes in the conformation of the protein, from $\alpha$ helical to disordered. The more easily performed optical density measurements indicate the state of the heme–protein interaction. It is obviously of value to measure both parameters, when possible. (B) For the same reasons, we could study the state of the heme in denaturing carbon monoxide myoglobin, *i.e.*, in form D, as reflected by the oxidation of heme by dissolved $O_2$ to give hemin, which takes only from 0.01 to 0.1 sec in a solution of free carbon monoxide heme, but 1 hr or more in solutions of carbon monoxide myoglobin. Steinhardt *et al.* (1966) have studied the denaturation of carbon monoxide hemoglobin (COHb) in the absence of oxygen. Their work showed that the initial rate of denaturation of COHb is not affected by the exclusion of oxygen. Their conclusions as to the sequence of events in the presence of oxygen are the same which we reach regarding the denaturation of carbon monoxide myoglobin. (C) Species D* is the final product of the reaction and can therefore be studied easily. Because of the eventual return of the spectrum of metmyoglobin to a value typical of the native molecule, even after a long period under denaturing conditions, we suspected that aggregation of the denatured material might cause the major difference between species D and D*. Earlier work indicated that both hemin (Kriste *et al.*, 1969) and denatured myoglobin (Hermans *et al.*, 1969) were suspect in this connection. Also, Polet and Steinhardt's (1969) work on hemoglobin points in this direction. We did some simple experiments using gel chromatography, whose results give a picture of the nature of species D*.

## Methods

Methods not described here were preformed as described in the preceding paper in this series. Carbon monoxide myoglobin working solutions were prepared by: equilibrating a metmyoglobin solution with 250-mm partial pressure of CO reducing this solution by adding a small amount of sodium dithionite (Fisher's reagent grade product) until the protein solution attains the characteristic red color, and passing the solution over a 1 × 20 cm mixed-bed resin (Bio-Rad Lab, type AG 501-X8) column. The dithionite-free carbon monoxide myoglobin solution was equilibrated with approximately equal pressures of CO and $O_2$. KCl was added to the solution to obtain 0.2 M ionic strength.

## Results

*Oxidation of Carbon Monoxide Heme.* Carbon monoxide heme dissolved in a solution containing a small amount of

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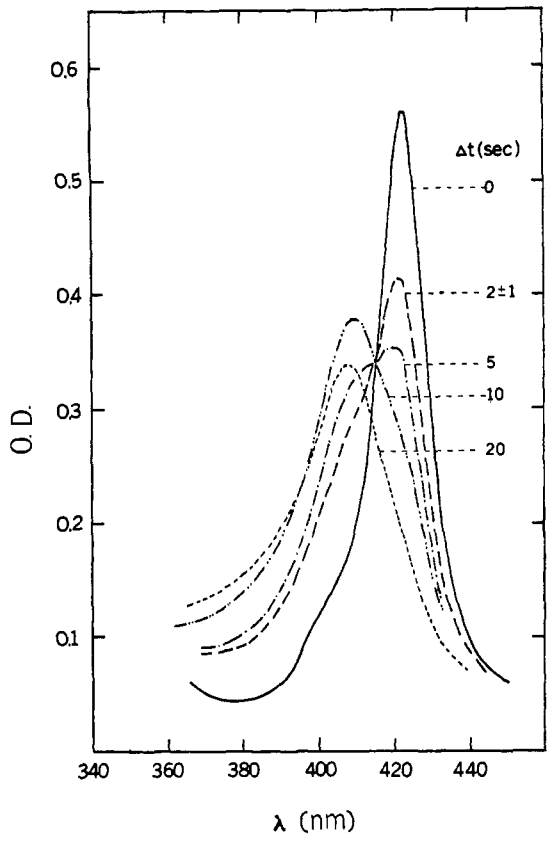


FIGURE 1: Spectra of refolded products of carbon monoxide myoglobin in the presence of oxygen. The protein has been unfolded for $\Delta t$ (seconds) with citrate–KCl buffer (pH 3.7) at 25°. Carbon monoxide myoglobin working solutions were free of dithionite ion and had been equilibrated with approximately 260- and 200-mm partial pressure of CO and $O_2$, respectively. Final concentration of carbon monoxide myoglobin was $3 \times 10^{-6}$ M. The protein was renatured at pH 5.5 for *ca.* 5 min.

dithionite is rapidly oxidized to hemin by mixing with buffer containing some dissolved air. We determined this by following the absorption at 407 nm in a stopped-flow experiment. The half-time for this reaction was about 30 msec at pH 4.5; since there was some dithionite in the mixture, we are not certain what is exactly the limiting factor in the process; in any case these results present an upper limit for the time needed for this oxidation.

*Kinetics of Denaturation of Carbon Monoxide myoglobin.* We have measured the time dependence of the spectrum at pH 3.73. Kinetic plots demonstrate two and sometimes three stages. In all, the behavior is very similar to that of metmyoglobin. At no instant do we observe in the spectrum absorption at 406 nm reminiscent of carbon monoxide heme. This suggests that the product of the denaturation is denatured metmyoglobin. This is confirmed by the data shown in Figure 1; namely, the spectra of carbon monoxide myoglobin solutions which were taken to pH 3.7 for periods $\Delta t$ from 0 to 20 sec in the presence of $O_2$, then brought back to pH 5.5 and left to renature for 5 min. For the first 10 sec there is an isosbestic point at the intersection of the spectra of carbon monoxide myoglobin and metmyoglobin, the peak at 423 nm decreases and another rises at 410 nm. The spectrum of the solution which had sojourned at low pH for 20 sec is depressed. Its shape and magnitude indicate that no carbon monoxide myoglobin is present, and that some of the denatured material has not been renatured.

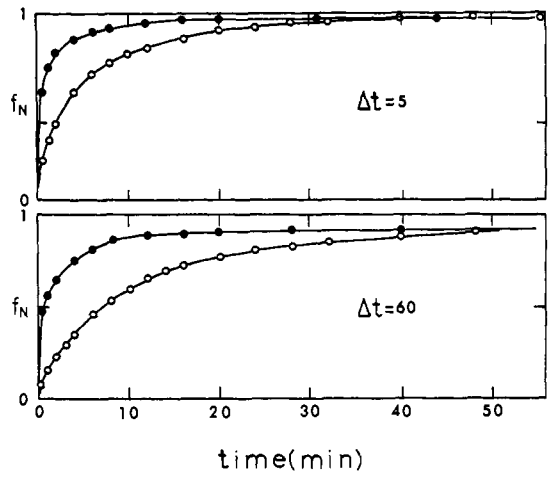


FIGURE 2: Kinetic traces of the refolding process of acid-denatured metmyoglobin. The protein had been denatured at pH 4.16 for (A) 5 min and (B) 60 min. The reactions were followed by the changes in optical rotation (●) and in optical density (○). The ordinate has been normalized to the fraction of native protein, $f_N$. The native material at neutral pH corresponds to $f_N = 1$.

*Kinetics of Unfolding Measured with Optical Rotation.* Only in the transition range are the rates low enough to perform meaningful measurements of the time dependence of the optical rotation. The measurements give essentially the same results as do those performed following the uv absorption, with a second stage of the reaction representing the reestablishment of the $N \rightleftharpoons D$ equilibrium accompanying the formation of form D* from D. These data are in agreement with the earlier evidence that the conversion N to D is a two state process.

*Kinetics of Refolding Measured with Optical Rotation.* We determined the refolding kinetics from form D* with optical rotation and optical density (Figure 2). The two methods clearly do not give coincident results, the formation of folded ($\alpha$ helical) protein being much more rapid than the regain of the heme absorption typical of native metmyoglobin. In contrast to what has recently been suggested for other proteins (Teipel and Koshland, 1971), we do *not* think that in this case the folded protein occurring before the absorption is reestablished is necessarily an erroneously folded conformation (see further below).

*Gel Chromatography of Denatured Myoglobin.* The results of molecular filtration studies on denatured metmyoglobin are shown in Figure 3. The elution volume of myoglobin denatured at pH 4.0 was identical to the void volume of the Sephadex G-200 column. The ratio of the absorptions at 280 and 370 nm is approximately the same as for the unfractioned mixture.

The elution pattern of denatured myoglobin at pH 3.01 is totally different. Two peaks were separated in this case. The ratio between the optical densities at 280 and 370 nm for the first peak eluted from the column is higher than the value of 0.50 for hemin in aqueous solution at pH 3. Hence this peak contains both heme and protein, in a molar ratio of approximately 2:1. The second elution peak is colorless indicating that it contains only globin.

Earlier work of Polet and Steinhardt (1969) on hemoglobin indicates that with this protein similar reactions occur. These authors used a column of Sephadex G-25 and worked at lower pH. Thus, these results are not directly comparable to ours. Our results on myoglobin are in good agreement

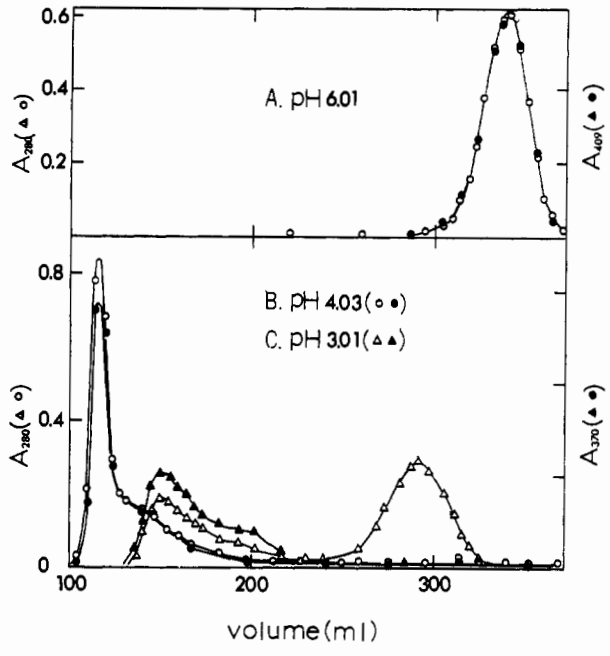


FIGURE 3: Elution pattern of myoglobin on a Sephadex G-200 column with citrate–KCl buffers at pH 6.01 (A), pH 4.03 (B), and pH 3.01 (C), ionic strenth = 0.2.

with the ultracentrifuge study of Kriste *et al.* (1969), except that our data show that the fraction of highest molecular weight contains both globin and heme.

## Discussion

The results indicate that the denatured species D is reversibly denatured myoglobin: the heme group is accessible to oxidation by oxygen, the protein has lost much of the $\alpha$-helical structure present in native myoglobin, to the same extent as on longer exposure to low pH (Beychok *et al.*, 1962) which, itself, is compatible with the demonstrated helix content of isolated peptides of myoglobin (Hermans and Puett, 1971) and of denatured heme-free myoglobin (apomyoglobin, Hermans *et al.*, 1969). It is formed in a reaction which is kinetically two-state in the transition region, and exists in a two-state equilibrium with native myoglobin.

All this is complicated by the fairly rapid formation of aggregates of hemin and denatured apomyoglobin. The experiments on refolding of this material do give rise to an interesting consideration. In returning the pH of the solutions to 5.5, we establish conditions where folded apomyoglobin is stable (Breslow, 1964). Correct folding of apomyoglobin (with regain of the optical rotation) requires dissociation of any associated materials and may thereby be relatively slow. The greater slowness of the regain of the absorption may simply be due to the even slower liberation of the heme from the complexes it is taking part in. It is of course also possible that the associated protein folds up partly, and thereby incorrectly, and that the formation of dissociated folded apomyoglobin molecules from these species is the slow step in forming myoglobin molecules. In this connection we mention some preliminary experiments which indicate that addition of the protein denaturant ethanol to the solution very markedly speeds up the refolding (as followed by the optical density regain) of material which has been denatured for a long time. Since ethanol is expected to solubilize the hemin molecules, this observation is in favor of the first alternative.

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