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Kinetics of the Reversible Reaction of Sperm Whale Myoglobin with Zinc*

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Kinetic measurements have been made on the suppression of Soret band intensity brought about by reaction of Zn ions with sperm whale ferrimyoglobin and on its reversal by diluting or sequestering the metal ion or by simply lowering the *p*H from 6.4 to a value less than 6. These measurements indicate a three-step process: (1) labilization of the protein structure by binding of Zn ions to sites on the surface of the macromolecule; (2) unfolding of the weakened structure, at least to a limited extent, concomitant with binding of a single Zn ion to a critical and otherwise inaccessible site, as the *rate-controlling* step; and (3) rapid polymerization of the Zn-reacted myoglobin such that interaction between the ferriheme moieties of the polymer suppresses and broadens the Soret band. The second step can be hastened by denaturing agents like alcohol. It is proposed that in the *rate-controlling* step the Zn ion ruptures the Fe^{3+}-F8 imidazole linkage and occupies the F8 imidazole group. Mutual binding of the Zn ion by the F8 and groups like the distal E7 imidazole group is not precluded. Sedimentation analyses of acid-denatured ferrimyoglobin show a large proportion of globin and a relatively small amount of protein aggregate. The latter fraction contains most, if not all, of the ferriheme. In this case, also, suppression and broadening of the Soret band is attributed to interaction between the ferriheme moieties of the aggregate.

Recently (Cann, 1963) it was shown that reaction of Zn ions with sperm whale ferrimyoglobin at *p*H 6.4 causes major changes in the ultraviolet and visible absorption spectra of the protein. It was concluded that Zn-reacted myoglobin is conformationally quite different from the unreacted protein. Reaction of the protein with Zn can be reversed to yield renatured ferrimyoglobin by one of the following three methods: (1) lowering the Zn concentration by dilution; (2) sequestering the Zn ions at *p*H 6.4 with EDTA[1] or citrate; or (3) lowering the *p*H of the reaction mixture to a value of 5.2. The renatured protein can be readily crystallized in the same crystal habit as ferrimyoglobin never exposed to Zn.

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[1] Abbreviations used in this work: EDTA, ethylenediaminetetraacetate; BAEE, benzoyl-L-arginine ethyl ester.

The most characteristic spectral change (Fig. 1) is a marked reduction in the Soret band intensity accompanied by a shift of the band from an absorption maximum of 408 mμ to one at 390 mμ. Whereas the former band is quite sharp, the latter is diffuse. Breslow and Gurd (1963) have described very similar spectral changes brought about by reaction of ferrimyoglobin with Cu^{2+} and have suggested that such changes are indicative of alterations in the ferriheme-protein linkage.

As shown by Figure 1, suppression of the Soret band by Zn proceeds at a measurable rate, and solutions of partially reacted protein apparently contain only two classes of absorbing species. The present communication describes kinetic measurements on the reaction and its reversal. These measurements indicate the three-step process that is summarized in the abstract.

The realization that binding of Zn ions by myoglobin mediates conformational changes has important implications, not only for methods of fractionation of biological materials, but also for the mechanisms of biochemical reactions such as the metal-activation and

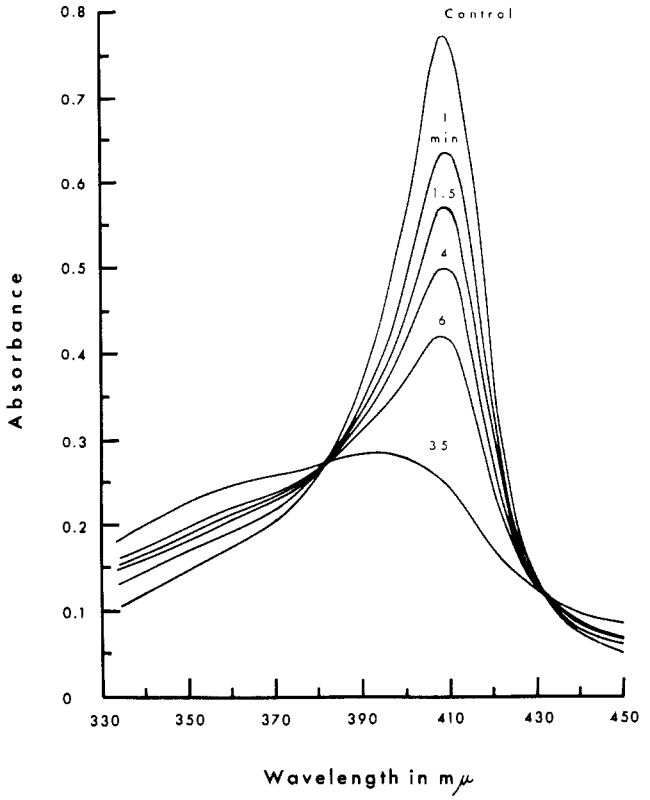


FIG. 1.—Alteration of the Soret band of ferrimyoglobin by reaction with zinc. 0.009% protein in 9×10^{-3} M $ZnAc_2$ + 0.04 M KCl, *p*H 6.4; 36°.

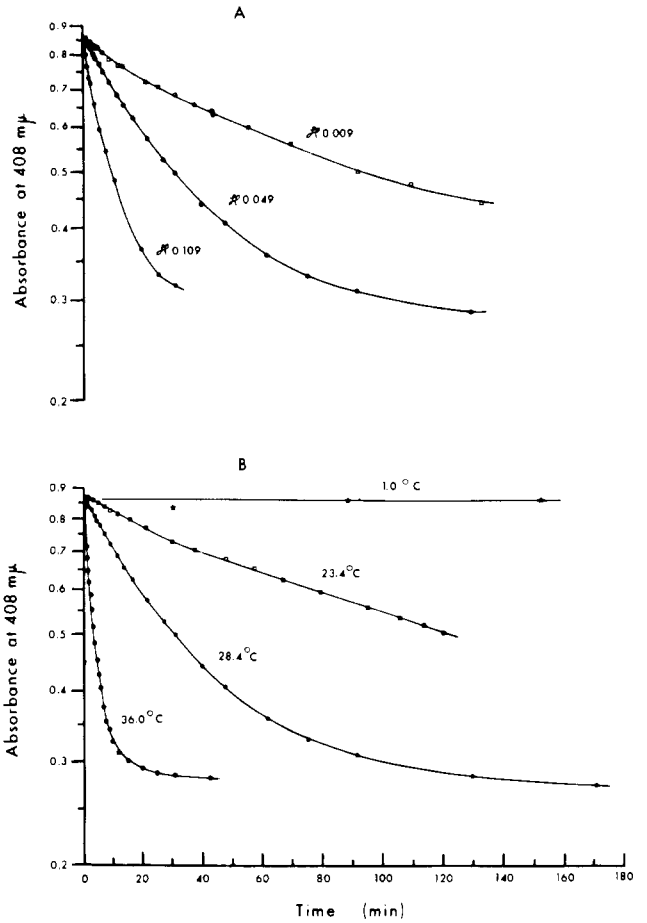


FIG. 2.—Time course of suppression of Soret absorption by 9×10^{-3} M $ZnAc_2$, *p*H 6.4. Semilogarithmic plot of absorbance at 408 mμ versus time in minutes: A, effect of ionic strength, $\mathcal{I}$; B, effect of temperature at ionic strength 0.049. Ferrimyoglobin concentration, 0.01%; ionic strength adjusted with KCl.

poisoning of enzymes. Myoglobin is an ideal protein for studying interaction with metal ions since its exact three-dimensional structure has been largely established by the crystallographic X-ray analyses of Kendrew and co-workers (1961), thereby providing the fundamental information required for a detailed description of the mechanism of its reactions.

EXPERIMENTAL

Solutions of Manns' sperm whale ferri(met)myoglobin were dialyzed against distilled water in the cold and lyophilized. Reaction mixtures were prepared by addition of 9 ml of temperature-equilibrated $ZnAc_2$-HAc-KCl solution (Ac symbolizes acetate ion) to 1 ml of salt-free myoglobin solution. An aliquot was immediately transferred to a thermostated cuvet in a Beckman DU spectrophotometer; and the change in absorbance, $A = \log_{10}(I_0/I)$, with time measured. In some experiments (Figs. 1 and 9) reaction was allowed to proceed in a thermostated bath; at various times aliquots were rapidly cooled to 0° to stop the reaction, and the spectra were recorded using a DK-2 spectrophotometer thermostated at about 10°. The preparation of myoglobin used in the experiments shown in Figure 6 reacted slower at 28.4° than did the other preparations. The difference may be related to the heterogeneity of myoglobin in solution (Edmundson and Hirs, 1962).

Ionic strength was calculated assuming the average composition of the zinc to be $ZnCl^+$ or $ZnAc^+$ (Bjerrum, 1950; Leden, 1941).

Stock solutions of Zn-reacted myoglobin for kinetic measurements on the reverse reaction (which were carried out with the same precautions as the forward reaction), tryptic digestion, and ultracentrifugal analyses were prepared by incubating Zn-containing solutions of ferrimyoglobin at 46° for 10 minutes followed by centrifugation at 20,000 rpm to remove a trace of insoluble material.

Sedimentation experiments were carried out in the Spinco Model E ultracentrifuge using ultraviolet optics with a 30-mm cell for 0.01% protein and schlieren optics for 0.3%. Identification of the schlieren peak with which the color sedimented was made by direct observation or photographically on metallographic plates, and by fractionation in a partition cell. Eastman 103-F plates were used to obtain the clearest photographic records. Kel-F cells were used for acid-denatured material. Molecular weights were determined at 8225 rpm by the approach to equilibrium method of Archibald (1947) as practiced by Ginsburg *et al.* (1956).

Carbonmonoxyhemoglobin was prepared from fresh, heparinized human blood by lysing washed red cells with distilled water and shaking the crude hemoglobin solution with toluene. After clarification by low-speed centrifugation, the solution was saturated with CO, subjected to high-speed centrifugation, and then resaturated with CO.

Ferrihemoglobin was prepared by oxidation of carbonmonoxyhemoglobin with a minimum amount of potassium ferricyanide, 8×10^{-3} M for 4% protein. Immediately following completion of oxidation as judged spectroscopically, the ferrihemoglobin solution was freed from ferro- and ferricyanide on a Sephadex G-25 column (2 × 22 cm), using 0.1 M KCl as eluent. The column was pretreated for 15 hours with 3×10^{-2}

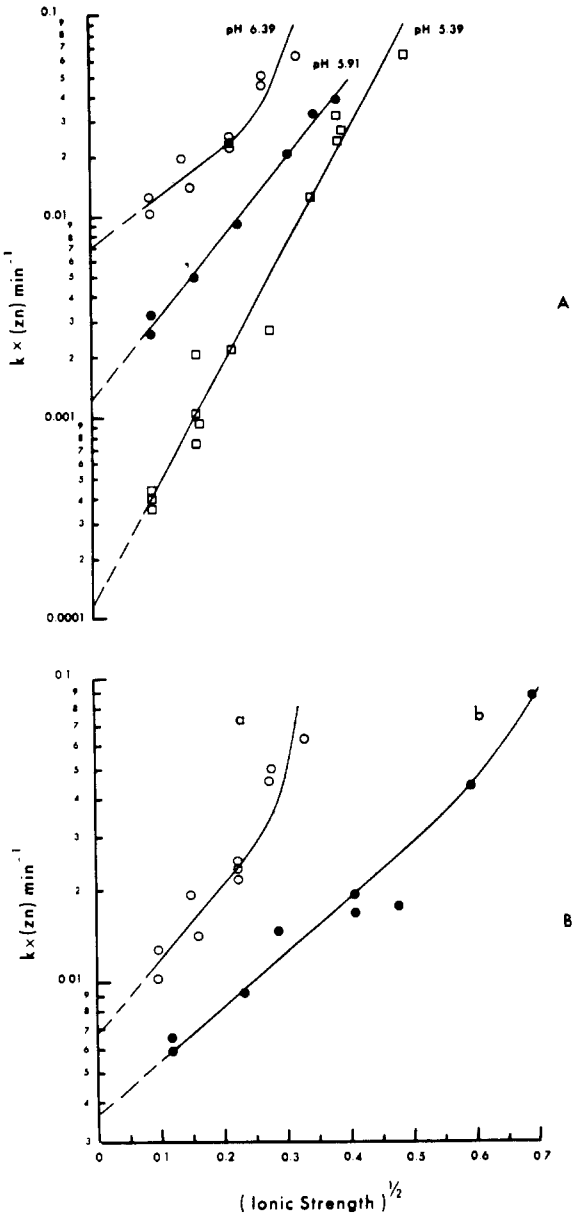


FIG. 3.—Primary salt effect on the rate of reaction of 0.01% ferrimyoglobin with Zn at 28.4°. Semilogarithmic plot of logarithmic rate, k(Zn) min^{-1}, versus square root of ionic strength: A, effect of pH for 9×10^{-3} M $ZnAc_2$; B, effect of zinc concentration at pH 6.4 (a) 9×10^{-3} M $ZnAc_2$, (b) 4.5×10^{-3} M $ZnAc_2$. Ionic strength adjusted with KCl.

M potassium ferricyanide, which was then eluted with 0.1 M KCl. The protein did not denature on the pretreated column.

Recrystallized hemin and BAEE were obtained from Nutritional Biochemicals Corp. The trypsin was Worthington's 2× crystallized, salt-free lyophilized material.

Forward Reaction

Order of Reaction and Effect of Ionic Strength.—Representative measurements on the rate of suppression of Soret band intensity due to reaction of 0.01% ferrimyoglobin with 9×10^{-3} M zinc ion at pH 6.4 are presented in Figure 2, which is a semilogarithmic plot of absorbance at 408 mμ versus time in minutes. Although the process follows a somewhat complex kinetic course,[2] the plots are linear during early stages of reaction so that initial logarithmic

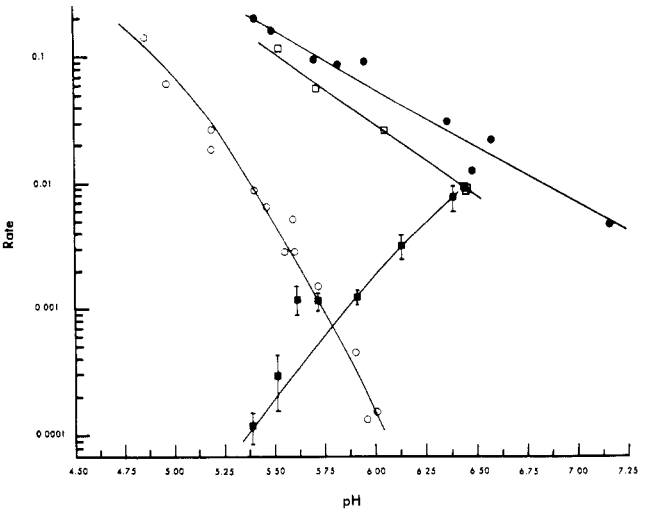


FIG. 4.—Dependency of rate of forward and reverse reaction upon pH at 28.4°. Semilogarithmic plot of rate versus pH: ‡, extrapolated logarithmic rate of reaction $[k(\text{Zn})]_{\mathfrak{C}=0}$ min^{-1} of 0.01% ferrimyoglobin with 9×10^{-3} M $ZnAc_2$, vertical lines through points represent ±2 standard errors of estimate, curve is theoretical one discussed in text; □, initial rate of reversal, $(dA/dt)_i$ min^{-1}, of 0.01% Zn-reacted protein by dilution; ●, initial rate of reversal of 0.005% Zn-reacted protein by 5×10^{-3} M EDTA; ○, initial rate of reversal of 0.01% Zn-reacted protein by hydrogen ions, curve is theoretical one discussed in text.

rate constants were obtainable. The value of the rate constant at ionic strength 0.049 M was found to drift only 14% over a 4-fold range of protein concentration, which shows that the reaction is first order with respect to protein.

In accordance with the predictions of the Brønsted-Debye-Hückel theory (Amis, 1949) of the primary salt effect on the kinetics of reaction between positively charged ions in solution, the rate of suppression of Soret band intensity increases with increasing ionic strength (Fig. 2A), and semilogarithmic plots of rate constant versus square root of ionic strength (Fig. 3) permit linear extrapolation of rate constants to zero ionic strength. Comparison of extrapolated rate constants at 9×10^{-3} and 4.5×10^{-3} M zinc (Fig. 3B) shows that the reaction is first order in zinc ion. This result was confirmed over a 50-fold range of zinc concentration in the presence of urea (see *Synergistic Effect of Urea or Ethanol*). It is concluded, therefore, that the *rate-controlling* step in suppression of Soret band intensity involves reaction of a single zinc ion with a single molecule of ferrimyoglobin. For such a reaction, the slopes of the Brønsted plots in Figure 3 are predicted to equal $0.98 Z_p Z_M$, where Z_p is the charge on the protein and Z_M that on the zinc. Since the average composition of the zinc is probably closer to $ZnCl^+$ or $ZnAc^+$ than Zn^{2+} (Bjerrum, 1950; Leden, 1941), the slope should simply equal $0.98 Z_p$ and, in fact, values of Z_p thus calculated increase with decreasing pH and agree with values of the titration charge on the protein (Breslow and Gurd, 1962) to within 18 ± 9%.

[2] At ionic strength 0.049 and 0.109 M, reversible first-order kinetics are followed during 70–90% of the time-course of reaction. However, for conditions where the reaction proceeds more slowly, as at ionic strength 0.009 M, the reversible first-order relationship holds for only the first 10% of reaction. Nor are second-order kinetics obeyed. Failure to follow simple kinetics may be due to the heterogeneity of myoglobin in solution (Edmundson and Hirs, 1962).

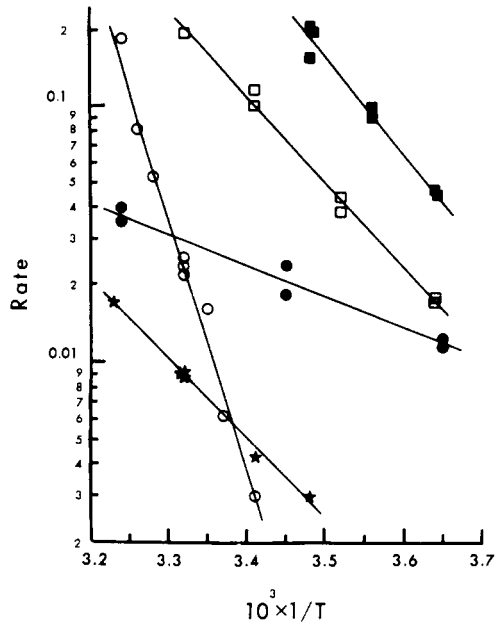


FIG. 5.—Arrhenius plot of logarithm of rate of reaction versus reciprocal of the absolute temperature. ○, logarithmic rate of reaction, k(Zn) min^{-1}, of 0.01% ferrimyoglobin with 9×10^{-3} M $ZnAc_2$, ionic strength 0.049 M and pH 6.4; ★, initial rate of reversal, $(dA/dt)_i$ min^{-1}, of 0.01% Zn-reacted protein by dilution at pH 6.4; □, initial rate of reversal of 0.005% Zn-reacted protein by 2.45×10^{-2} M citrate at pH 6.4; ■, initial rate of reversal of 0.005% Zn-reacted protein by 2.5×10^{-2} M EDTA at pH 6.4; ●, initial rate of reversal of 0.01% Zn-reacted protein by hydrogen ions at pH 5.15.

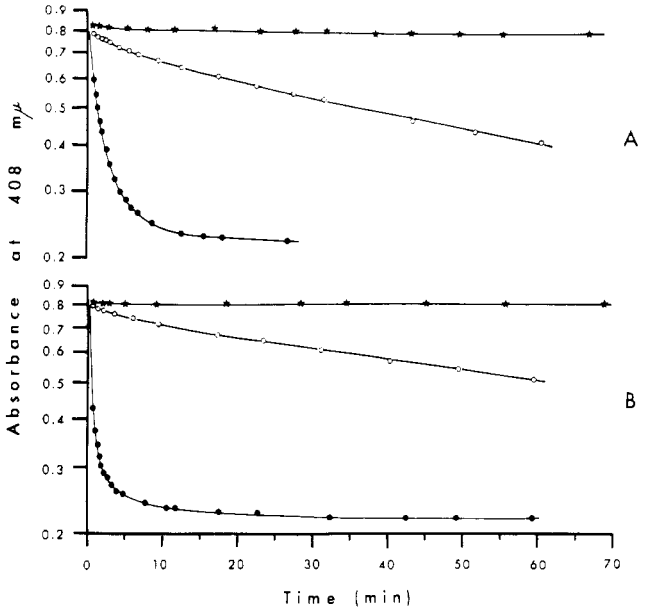


FIG. 6.—Synergistic effect of urea (A) or ethanol (B) on reaction of ferrimyoglobin with zinc. Semilogarithmic plot of absorbance at 408 mμ versus time in minutes. A: ★, 3.6 M urea + 9×10^{-3} M NaAc + 0.04 M KCl, pH 6.40; ○, 9×10^{-3} M $ZnAc_2$ + 0.04 M KCl, pH 6.45; ●, 3.6 M urea + 9×10^{-3} M $ZnAc_2$ + 0.04 M KCl, pH 6.42. B: ★, 8×10^{-3} M NaAc + 0.03 M KCl in 10% ethanol, pH 6.40; ○, 8×10^{-3} M $ZnAc_2$ + 0.032 M KCl, pH 6.45; ●, 8×10^{-3} M $ZnAc_2$ + 0.032 M KCl in 10% ethanol, pH 6.45. Protein concentration, 0.009%; temperature 28.4°.

Effect of pH.—As shown in Figure 3A, the rate of reaction is rather sensitive to hydrogen-ion concentration, the value of the extrapolated rate constant decreasing by a factor of 60 when the pH value is lowered from 6.39 to 5.39. This result is most simply interpreted as indicating that the *rate-controlling* reaction involves two ionizable groups, presumably imidazole, on the protein molecule and suggests the kinetic scheme:

$$PH_n + 2\,H^+ \rightleftharpoons PH_{n+2} \quad (1)$$

$$PH_n + Zn \longrightarrow DH_nZn \quad (2)$$

where PH_n and PH_{n+2} represent two protonated forms of native ferrimyoglobin, and DH_nZn the Zn-reacted protein which, as shown later, possesses a different macromolecular configuration than the unreacted protein. Reaction (2) is assumed to be rate limiting. This scheme leads to the following expression for the initial logarithmic rate of decrease of absorbance:

$$\left(\frac{d \ln A}{dt}\right) = -\left(\frac{\epsilon_1 - \epsilon_2}{\epsilon_2}\right) k_0(\mathrm{Zn})/[1 + K(\mathrm{H})^2] = -k(\mathrm{Zn}) \quad (3)$$

where k_0 is the specific rate of reaction (2), K is the equilibrium constant of reaction (1), and ϵ_1 and ϵ_2 are the molar extinction coefficients of native and Zn-reacted protein, respectively. Experimentally determined values of k(Zn) extrapolated to zero ionic strength are plotted versus pH in Figure 4. The curve is a theoretical one computed for $(\epsilon_1 - \epsilon_2/\epsilon_2)k_0$-(Zn) taken to be equal to 2.04×10^{-2} min^{-1}, and K, 10^{13} liter^2 mole^{-2} (two ionizable groups each with $pK = 6.5$). The values of the two constants were selected with the aid of a plot of experimental values of $1/k$(Zn) versus $(H)^2$. Considering the narrow range of pH values, the theoretical curve is a reasonable description of the dependency of the reaction rate upon pH.

Temperature Dependence.—As illustrated in Figure 2B, the rate of reaction is very sensitive to temperature. Whereas the reaction does not go at 1°, it proceeds to completion in about 30 minutes at 36°. The activation energy, obtained from the Arrhenius plot shown in Figure 5, is 46 kcal mole^{-1}. Clearly, a reaction with such a high activation energy can proceed at an appreciable rate at room temperature only if the entropy of activation is large and positive. A value of 84 eu for the entropy of activation was calculated from the value of the specific rate constant k derived from the theoretical curve shown in Figure 4. Such large values for the energy and entropy of activation are typical of protein denaturation and indicate that reversible macromolecular conformational changes occur concomitantly with the rate-controlling reaction of a single Zn ion with some critical site on the protein molecule.

Evidence for Reversible Conformational Changes.—Supporting evidence for conformational changes was sought in both difference-ultraviolet-absorption spectra and alterations of chemical properties. The results of the spectroscopic experiments have been presented previously (Cann, 1963). It need only be mentioned here that, as in the case of native versus acid-denatured myoglobin which is known to be extensively unfolded (Beychok *et al.*, 1962), the difference spectrum of unreacted versus Zn-reacted ferrimyoglobin shows a prominent peak at 228 mμ, which may arise largely from a change in peptide-bond absorptivity concomitant with a conformational change (Glazer and Smith, 1960; Wetlaufer, 1962).

Zinc-reacted ferrimyoglobin has also been judged to be structurally altered by the classical criteria of protein denaturation, e.g., drastically decreased solubility in salt solutions and increased susceptibility to

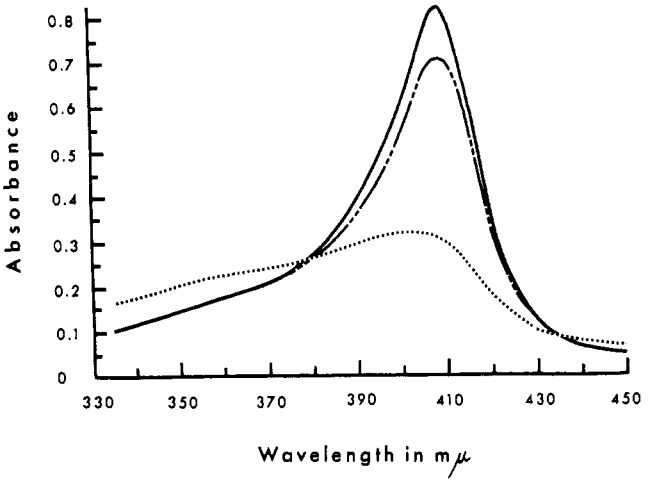


FIG. 7.—Synergistic effect of ethanol on reaction of ferrimyoglobin with cadmium. ———, 8×10^{-2} M NaAc + 0.04 M KCl in 10% ethanol, *p*H 6.4, incubated 1 hour at 36°; — · —, 9×10^{-2} M $CdAc_2$ + 0.04 M KCl, *p*H 6.4, incubated 1 hour at 36°; ·····, 9×10^{-2} M $CdAc_2$ + 0.04 M KCl in 10% ethanol, *p*H 6.4, incubated 20 minutes at 36°. Protein concentration, 0.0095%.

tryptic digestion. Whereas native myoglobin is very soluble in salt solutions, e.g., solubility of 3 mg/ml even in ionic strength 8 M $(NH_4)_2SO_4$, the Zn-reacted protein is completely precipitated from its *p*H 6.4 solution containing 2.5 mg protein/ml by only 0.25 M NaCl. The solubility properties of the native protein are recovered when reaction with Zn is reversed. Furthermore, the renatured protein crystallizes in the same crystal habit as myoglobin never exposed to Zn.

Sensitivity to tryptic digestion was determined grossly by measuring the extent of production of trichloroacetic acid–soluble material under a given set of conditions. One ml of solution containing 5 mg of either native or Zn-reacted myoglobin and 0.045 mg trypsin was incubated at 36° for 4 hours. In the case of native myoglobin the electrolyte composition was 9×10^{-3} M NaAc + 0.04 M KCl, *p*H 6.4; with Zn-reacted protein, 9×10^{-3} M $ZnAc_2$ + 0.04 M KCl, *p*H 6.4. Following incubation at 36°, 3 ml of 5% trichloroacetic acid was added to the digestion mixture, the slurry was clarified by centrifugation, and the absorbance of the supernatant was determined at 280 mμ, using as a blank the appropriate control solution which contained trypsin alone and which was carried through the same procedure as the digestion mixture. Zero-time controls were also included. In five experiments the average change in absorbance of the supernatant after 4 hours' digestion was 0.027 ± 0.027 in the case of the native protein and 0.232 ± 0.027 for the Zn-reacted protein. (The ratio of absorbances after 2 hours of incubation was only 10% less than that after 4 hours.) Addition of Zn to supernatants from native protein–trypsin digestion mixtures did not enhance their absorbances. Apparently under the conditions used in these experiments the limited digestion observed is essentially completed in 4 hours, since addition of fresh trypsin to digestion mixtures after this time followed by a further 4-hour incubation did not increase the absorbances of the subsequent supernatants over that found after the first 4 hours. The possibility that the increased sensitivity of Zn-reacted myoglobin to tryptic digestion might be an artifact arising from some action of Zn on trypsin, e.g., protection against self-digestion, was eliminated by measuring the esterase activity of trypsin on the synthetic substrate BAEE in the presence and absence of Zn, using the spectrophotometric method of Schwert and Takenaka (1955). Electrolyte compositions and *p*H were the same as in the case of myoglobin. Reaction mixtures containing 5×10^{-4} M BAEE and 0.016 mg trypsin/ml were incubated for 2 hours at 36°. The resulting change in differential absorbance at 250 mμ was the same in the presence as in the absence of zinc, 0.446 ± 0.012 and 0.442 ± 0.016, respectively.

Synergistic Effect of Urea or Ethanol.—A very strong synergism in suppressing Soret intensity has been observed between Zn and urea or ethanol. The results of a typical experiment on the synergistic effect of urea are presented in Figure 6A, which shows the time-course of suppression of Soret band intensity brought about by exposure of the protein to 3.6 M urea, 9×10^{-3} M Zn, and the combination of 9×10^{-3} M Zn and 3.6 M urea, each at the same *p*H and ionic strength. Whereas the rate of decrease of absorbance due to the denaturing action of urea is very much smaller than that brought about by Zn, the rate due to the combined action of Zn and urea is more than an order of magnitude greater than that due to Zn alone. Even 0.9 M urea, which alone has no effect on absorbance, produces a 2.5-fold synergistic enhancement of the rate of reaction. In experiments where the protein was first exposed to either Zn or urea 10 minutes prior to addition of the other reagent, there was an abrupt change in kinetics from low to very high rate of reaction immediately upon addition of the second reagent. The lack of a measurable time lag shows that the combined action of Zn and urea is truly mutual. The synergistic rate of reaction is first order in Zn over a 50-fold range of Zn concentration (determined in 7.2 and in 3.6 M urea) and about 1.6 order in urea over the concentration range 7.2–0.9 M (determined in 9×10^{-3} M Zn). The latter result is in contrast to urea denaturation in the absence of Zn, which is generally a very high-order reaction; a few exploratory measurements with myoglobin indicate at least a seventh and most likely greater order of reaction. Clearly, in the synergistic reaction urea is exerting its action on a very limited portion of the protein molecule. When one reflects upon the fact (Banerjee, 1959) that urea reacts with hemin to form hemin-$(urea)_2$ and replaces bases in ferrihemochromes, e.g., in hemin-pilocarpinate, the conclusion seems reasonable that the synergistic action of urea is on the Fe^{3+}-F8 imidazole bond.

The synergistic effect of ethanol is illustrated in Figure 6B. Under the conditions of our experiments, exposure of myoglobin to 10% ethanol does not alter Soret absorption. However, the combination of 10% ethanol with 8×10^{-3} M Zn suppresses absorption at a rate fifty times greater than that due to Zn alone. The effect is, perhaps, even more striking in the case of cadmium which also suppresses the Soret band but only at much higher concentrations than does Zn. Thus, for example, reaction of myoglobin with 9×10^{-3} M Cd for 19 hours at 36° produces only a 5% decrease in absorbance; and it requires 1 M Cd to give the same effect as 9×10^{-3} M Zn. However, the action of Cd is greatly enhanced by addition of ethanol as illustrated in Figure 7 for 9×10^{-2} M Cd, which alone causes only a 16% decrease in absorbance in 1 hour at 36°, but in conjunction with 10% ethanol almost complete suppression in 20 minutes. These results indicate that Zn and Cd react with some critical site which is most likely not on the surface of the protein molecule but, rather, buried and protected from reaction by steric hindrances which can

be overcome only by changes in macromolecular conformation. Such changes would be greatly enhanced by the denaturing tendency of ethanol.[3]

Spectral Change and State of Aggregation.—The Zn-mediated alterations in the Soret band (Fig. 1) are on first consideration surprising, since it is difficult to visualize how major spectral changes could arise from changes in protein configuration per se, even if accompanied by rupture of the Fe^{3+}-F8 imidazole linkage. On the other hand, broadening of the Soret band of hematin due to aggregation in alkaline aqueous solution is well known (Inada and Shibata, 1962); but the spectral changes are not nearly as marked as with Zn-reacted myoglobin. It seemed more to the point in our case to make measurements in solutions containing an organic solvent such as acetone (Fig. 8). In 50% acetone, 8.1×10^{-6} M hemin shows a sharp Soret band at 400 mμ with an extinction coefficient which agrees to within 20% with that for monomeric hematin in aqueous solution (Inada and Shibata, 1962). Some broadening of the peak and a blue shift occurs in 100% acetone; but the truly drastic spectral changes produced by aggregation of the hemin when the acetone concentration is lowered to 10% are very similar to those brought about in myoglobin by Zn. As reported previously (Cann, 1963), 0.01% Zn-reacted myoglobin is aggregated as revealed by a sedimentation constant of about 4 S as compared to 2 S for unreacted protein. As shown in Table I, the Zn-reacted protein is a mixture of extremely assymmetrical polymers with a weight-average molecular weight of about 160,000 at a protein concentration of 0.3%. Velocity ultracentrifugal patterns of 0.3% Zn-reacted protein show a single sedimenting peak, 4.25 S, with which the color is also associated. When reaction with Zn is reversed, the sharp Soret band is recovered and the sedimentation constant returns to its native value, indicating depolymerization into monomeric units. The conclusion seems justified, therefore, that suppression and broadening of Soret absorption is due to interaction between the ferriheme groups within the polymers of Zn-reacted myoglobin.[4]

TABLE I

MOLECULAR WEIGHTS OF Zn-REACTED MYOGLOBIN[a] DETERMINED DURING THE APPROACH TO SEDIMENTATION EQUILIBRIUM

Time of Sedimentation (min)	Molecular Weight[b,c] Meniscus	Bottom
52	180,000	150,000
68	150,000	160,000
84	160,000	160,000
100	120,000	190,000
116	130,000	210,000
132	110,000	210,000
148	110,000	210,000
164	86,000	240,000
228	93,000	240,000

[a] 0.3% Zn-reacted myoglobin in 9×10^{-3} M $ZnAc_2$, ionic strength 0.049 and *p*H 6.4; sedimentation constant, $s_{20,w}$, of 4.25 S. Native myoglobin at same protein concentration and ionic strength has $s_{20,w}$ = 2.02 S. [b] The value of $\bar{V}$ taken to be the same as that (0.741) for native myoglobin. [c] The weight-average molecular weight of about 160,000 and $s_{20,w}$ = 4.25 S give a frictional ratio of 2.36, which corresponds to an axial ratio of 30 for an unhydrated prolate ellipsoid or 22 if 30% hydration is assumed.

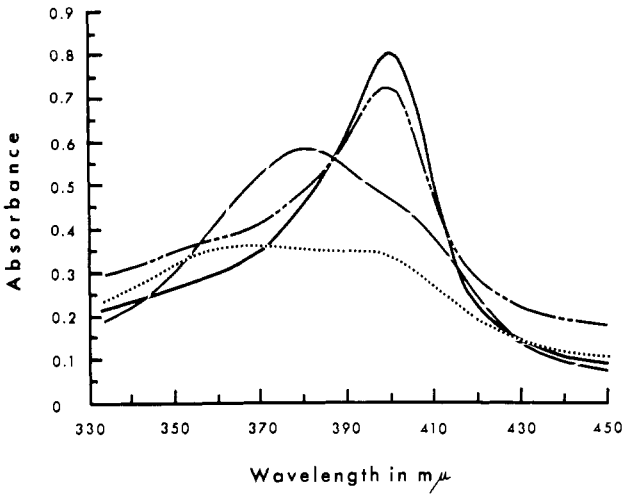


Fig. 8.—Spectrum of hemin. —, 8.1×10^{-6} M hemin in 50% acetone,— —, in 100% acetone, ---------, in 10% acetone; —·—·—, 8.1×10^{-7} M hemin in 10% acetone. Measurements made 10 minutes after preparation of solutions from a stock in 100% acetone. Path length: 1 cm for 8.1×10^{-6} M hemin; 10 cm for 8.1×10^{-7} M hemin.

A similar suppression of Soret absorption accompanies acid denaturation of ferrimyoglobin (Breslow and Gurd, 1962). With our material in 0.04 M KCl the spectral change was 40% complete at *p*H 4.03, 87% at *p*H 3.88, and complete at *p*H 3.53. Ultracentrifugal analyses, Table II, show that aggregation is likewise involved. The solutions contained a major component with a lower sedimentation constant than native myoglobin and a minor one with a sedimentation constant considerably larger than native. The color sedimented with the faster component as judged by visual and photographic observation as well as by fractionation and analysis using either a partition cell or a standard analytical one. (In the latter experiment the faster component was sedimented to the bottom of the cell, the rotor coasted to a stop, and 0.3 ml solution was withdrawn from the cell.) Fractions of the slower sedimenting material, 0.083–0.14% protein by Folin, had a Soret absorbance which was only 20% of that expected of acid-denatured protein and even this small absorbance appeared to be due to contamination during sampling. These

TABLE II

ULTRACENTRIFUGAL COMPOSITION OF 0.3% FERRIMYOGLOBIN IN 0.04 M KCl AT 21°

*p*H	Composition
6.20	100% 2.1 S
3.85	80% 1.6 S
	20% 3.1 S
3.55	65% 1.5 S
	35% 4.6 S
2.70	87% 1.7 S
	13% 4.2 S

[3] Dimethylformamide (DMF) at a concentration of 1.3 M also exerts a strong synergistic effect, increasing by 20-fold the rate of Zn-induced suppresssion of absorption; but a decision as to mechanism cannot be made at this time. At sufficiently high concentration, DMF denatures myoglobin as judged by loss of solubility in water; but the denatured protein is colored red, which suggests that DMF reacts with the heme iron.

[4] From preliminary ultracentrifuge experiments of Breslow and Gurd (1963) it can be inferred that myoglobin spectrally altered by reaction with Cu^{2+} is also aggregated.

results indicate that acid liberates the ferriheme moiety from the protein part of myoglobin and that the freed ferriheme is sequestered by a relatively small amount of aggregated protein. Interaction between the ferriheme groups in the aggregate is held responsible for suppression and broadening of the Soret band.

Reverse Reaction

Reversal by Dilution.—Suppression of Soret band intensity by reaction of myoglobin with 9×10^{-3} M Zn at *p*H 6.4 apparently proceeds to completion and the question of reversibility naturally arises. That the reaction is, in fact, reversible was demonstrated by diluting the Zn after completion of the forward reaction and observing the change in absorbance at 408 mμ. In a typical experiment, a solution of 0.5% Zn-reacted ferrimyoglobin in 9×10^{-3} M $ZnAc_2$ + 0.04 M KCl, *p*H 6.4, was diluted 1/50 at 28.4° with 0.02 M NaAc at *p*H 6.4 to give 0.01% protein and 1.8×10^{-4} M Zn. The immediate decrease in absorbance due to dilution of the protein was followed by an increase, at first linearly with time, but later at a progressively declining rate until a constant value of absorbance corresponding to approximately 60% reversal was attained after about 24 hours. Apparently a new equilibrium position was established at the lower Zn concentration since at *p*H 5.5 the reverse reaction proceeded to about 90% completion. The initial rate of the reverse reaction which has an activation energy of 14 kcal mole^{-1}, is approximately first order in protein. Quantitative comparison of these results, particularly the activation energy, with those obtained by other methods of reversal is made somewhat uncertain by the unusually high myoglobin concentration used in the preparation of the Zn-reacted protein. There is evidence that the degree of polymerization of the Zn-reacted protein increases with increasing protein concentration which introduces a possible complication of the contribution of the heat of depolymerization to the activation energy of the reverse reaction. Nevertheless the data are presented in Figures 4 and 5 to emphasize the reversibility of the reaction.

Reversal by Citrate or EDTA.—Reaction with Zn can also be reversed by sequestering the metal ion with citrate or EDTA. In the case of reversal by citrate, one volume of sodium citrate, *p*H 6.38, was added to one volume of 0.01% Zn-reacted ferrimyoglobin containing 9×10^{-3} M Zn at *p*H 6.38. The time course of reversal was similar to that described above for the dilution experiments; and the rate increased with increasing citrate concentration. Thus, when the concentration of citrate in the reaction mixture was 5×10^{-3} M, the reverse reaction proceeded to 70% completion in 4.25 hours, while 2.45×10^{-2} M citrate gave 80% reversal in 1.25 hour. The activation energy for citrate reversal (Fig. 5) is 15 kcal mole^{-1}.

Reversal with EDTA was carried out in the same manner as with citrate, the *p*H of the EDTA solution being adjusted so as to give reaction mixtures of the desired *p*H. The time course of reversal was generally the same as with dilution and citrate, and the rate increased with increasing concentration of EDTA. Whereas 5×10^{-3} M EDTA gave something less than 50% reversal in 1 hour at *p*H 6.48 and 28.4°, 2.5×10^{-2} M gave 90% reversal in less than 10 minutes. The reaction is also sensitive to *p*H. Thus, while less than 50% reversal occurred in 1 hour at *p*H 6.48 (5×10^{-3} M EDTA), 90% reversal was attained in less than 10 minutes at *p*H 5.4. The initial rate is first order in hydrogen ion (Fig. 4) and the data indicate the involvement of an acidic group having a *pK* in the range 5–4. The activation energy (Fig. 5) is 17 kcal mole^{-1}.

Reversal by Hydrogen Ions.—Perhaps the most informative method of reversing the suppression of Soret absorption by reaction with Zn is to lower the *p*H from 6.4 to a value less than 6. Complete reversal was achieved by 5.5-hour dialysis of a 0.01% solution of Zn-reacted protein (9×10^{-3} M $ZnAc_2$ + 0.04 M KCl) against 0.02 M NaAc buffer, *p*H 5.3. In kinetic experiments the *p*H was adjusted by addition of a very small volume of diluted acetic acid. The absorbance began to increase immediately upon lowering the *p*H and followed a time course similar to the other methods of reversal. The initial rate is first order in protein, has an activation energy of 5.6 kcal mole^{-1} (Fig. 5), is inhibited by Zn, and increases markedly with decreasing *p*H (Fig. 4). Whereas only a low percentage of reversal was achieved in 1.5 hour at *p*H 6.0 and 28.4°, the absorbance attained a constant value corresponding to about 75% reversal in 2 hours at *p*H 5.18 and 90% in 0.75 hour at *p*H 4.85, which in terms of initial rate of reaction represents almost a 1000-fold increase in rate. These observations, along with those on the other methods of reversal and on the forward reaction, suggest the kinetic scheme:

$$DH_iZn + 2\,H^+ \rightleftharpoons DH_{i+2} + Zn \qquad (4)$$

$$DH_{i+2} + H^+ \rightleftharpoons DH_{i+3} \qquad (5)$$

$$DH_{i+3} \longrightarrow PH_{i+3} \qquad (6)$$

where DH_iZn is a protonated form of the Zn-reacted protein, DH_{i+2} is a protonated form of a conformationally altered protein obtained by removing the Zn from the critical site, and PH_{i+3} is renatured protein. Reaction (6) is assumed to be rate limiting. This scheme leads to the following expression for the initial rate of recovery of Soret band absorbance:

$$\left(\frac{dA}{dt}\right)_i = \frac{(\epsilon_1 - \epsilon_2)k - K_1K_2(T)(H)^3}{[K_1K_2(H)^3 + K_1(H)^2 + (Zn)]} \qquad (7)$$

where K_1 is the equilibrium constant of reaction (4), K_2 is the equilibrium constant of reaction (5), k_- is the specific rate constant of reaction (6), and (T) is the total protein concentration. The curve shown in Figure 4 is a theoretical one for K_1 taken to be equal to 1.07×10^{10} liter mole^{-1} (product of the association constants for two ionizable groups of *p*K 6.5 and the dissociation constant (Tanford, 1952) of the Zn-imidazole bond, 10^{-3} mole liter^{-1}); for K_2, 10^4 liter mole^{-1}, which is consistent with the *p*H dependency of reversal by EDTA; and for k_-, 0.0231 min^{-1}. Clearly, the theoretical curve is a reasonable representation of the experimental data. Furthermore, the kinetic scheme correctly predicts the inhibitory effect of Zn, which was tested over a 4-fold range of Zn concentrations at several *p*H values. Plots of the reciprocal of initial rate versus concentration of Zn were approximately linear and had slopes which agreed to within 20–70% with those predicted by equation (7).

Two important conclusions can be drawn from comparison of the kinetics of reversal by the several methods described above. First, at a given *p*H value of 6 or lower the rate of reversal by diluting or sequestering the Zn ions is more than an order of magnitude greater than the rate of reversal by hydrogen ions (Fig. 4). It can be inferred from this that, although the *rate-controlling* step in suppression of Soret band intensity is first order in Zn, more than one Zn ion is

involved in the overall process. This is in accord with the finding of Breslow and Gurd (1963) that the binding of more than one or two Cu^{2+} ions by myoglobin is required for formation of spectrally altered protein. The second conclusion concerns the nature of the ionizable groups involved in the reverse reaction. One can surmise that reversal by both EDTA and hydrogen ions involves a carboxyl group of the protein and that in addition two imidazole groups are involved in reversal by hydrogen ions. In that event, the difference, 11 kcal $mole^{-1}$, in activation energies for the two modes of reversal should equal the difference in $\Delta H°$ for formation of Zn-EDTA complex, −4.5 kcal $mole^{-1}$ (Charles, 1954), and for protonation of two imidazole groups, −15 kcal $mole^{-1}$ (Breslow and Gurd, 1962). The agreement is remarkably good.

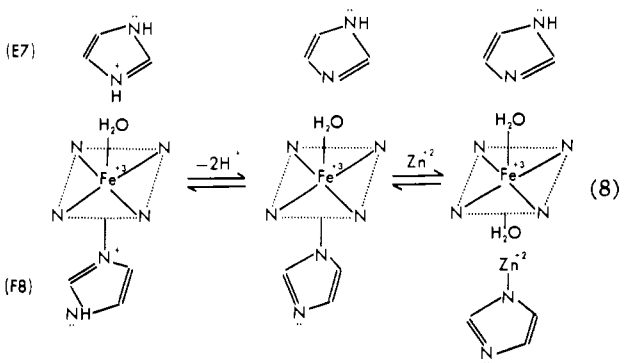
Comparison of Cu *with* Zn

As mentioned previously, Breslow and Gurd (1963) in an independent and contemporaneous study have found that reaction of myoglobin with Cu causes spectral alterations similar to those produced by Zn, and one supposes that the two reactions bear much in common mechanistically. However, comparison of Breslow and Gurd's Cu-binding and acid-base titration data with our kinetic experiments on Zn indicates that at least a quantitative difference must exist. This was verified by experiments which showed that suppression of the Soret band of myoglobin by reaction with Cu proceeds readily at the low *p*H values which cause reversal of the Zn-mediated reaction. Thus, for example, the spectral change proceeds to completion with 9×10^{-3} M Cu at a *p*H as low as 4.92. Even with 9×10^{-4} M Cu complete reaction is still realized at *p*H 5.23, and only about 60% reversal occurs when the *p*H is lowered to 4.68. When reaction with the lower concentration of Cu is carried to virtual completion at *p*H 5.04 and 46°, about 25% reversal of the spectral change occurs when the reaction mixture is cooled to room temperature.

Discussion

The following is a proposed mechanism whereby binding of Zn ions to ferrimyoglobin causes macromolecular conformational changes and alters drastically the strongest and most characteristic absorption band of heme-proteins. Evidently, binding of Zn ions to sites on the surface of the protein molecule labilized the macromolecular structure, presumably by perturbing electrostatic interactions thereby altering the delicate balance of forces which determine secondary and tertiary structure. In the *rate-controlling* step, the weakened structure is assumed to unfold, at least to a limited extent, concomitant with binding of a single Zn ion to a critical and otherwise inaccessible site. These ideas are consistent with the involvement of more than one Zn ion in the reaction even though the *rate-controlling* step is first order in Zn, with large energy and entropy of activation, large standard entropy of reaction,[5] the increased sensitivity of the Zn-reacted protein to tryptic digestion, synergistic effect of ethanol, which agent is expected to present additional stress to the macromolecular structure, and with the suprisingly low reactivity of Cd ion which could conceivably encounter greater steric encumbrances than the smaller Zn ion in reaching the critical site.

The implication of imidazole groups is clear and conforms to the chemical findings of Breslow and Gurd (1963) for binding of Cu ions to myoglobin. Imidazole groups, whose reaction with Zn might conceivably labilize structure, are those of the EF4, EF5, and FG3 histidyl residues, all of which groups are in the neighborhood of the ferriheme moiety (Kendrew, 1961; Kendrew *et al.*, 1961; Edmundson, 1963). The most probable critical site in the protein is the imidazole group of the F8 histidyl residue which is linked to the Fe^{3+} of the ferriheme moiety (Kendrew *et al.*, 1961; Kendrew, 1961). It is proposed that in the *rate-controlling* step a Zn ion ruptures the Fe^{3+}-imidazole bond and occupies the F8 imidazole group via the following scheme:



in which the planar structure involving four nitrogen atoms represents the protoporphyrin. In other words, it is postulated that both the ferriheme-bonded (F8) and distal (E7) imidazole residues must each dissociate a proton for rupture of the Fe^{3+}-imidazole bond by Zn ion to occur. This is consistent with the fact that the reactions of ferrimyoglobin with CN^- and of cyanoferrimyoglobin with H_3O^+ proceed about forty and twenty times faster, respectively, when the heme-linked group is in the state of ionization more favorable for coulombic attraction (George and Hanania, 1955). The possibility of mutual binding of the Zn ion by the F8 and groups like the distal E7 imidazole group is not precluded. When reaction with Zn is reversed by sequestering the Zn ions, the rate of reversal should be, as observed, independent of the state of ionization of the two imidazole groups. On the other hand, protonation of these groups should, and apparently does, play an important role in determining the rate of hydrogen-ion mediated reversal. Another ionizable group, possibly carboxyl, is involved in both modes of reversal, but there is no experimental basis for assessing its role. Finally, the model allows for synergistic rupture of the Fe^{3+}-F8 imidazole bond by urea via formation of Fe^{3+}-urea.

The model is also consistent with the relative reactivity of Zn and Cu, which are presumed to act via similar mechanisms. In that event, the fact that Cu suppresses Soret absorption at the low *p*H values which reverse the Zn-mediated reaction may simply be due to the much greater affinity of Cu for imidazole (Edsall and Wyman, 1958).

Reaction scheme (8) correctly predicts the dependency of the rate of suppression of Soret intensity by Zn upon the strength of the iron-ligand bonds, the weaker the bonds the greater the rate. Thus, ferrihemoglobin with its weaker bonds as judged from

[5] A value of 42 liters $mole^{-1}$ for the equilibrium constant is given by the ratio of specific reaction rate constants for the forward and hydrogen-ion mediated reverse reaction; a value of 25 kcal $mole^{-1}$ for $\Delta H°$, by the difference in activation energy after correction for protonation of two imidazole groups; and 90 eu for $\Delta S°$ by the second law. The similarity of $\Delta S°$ and $\Delta S^{\ddagger}$ values is striking and could mean that the conformation of the Zn-reacted protein is similar to that in the activated complex.

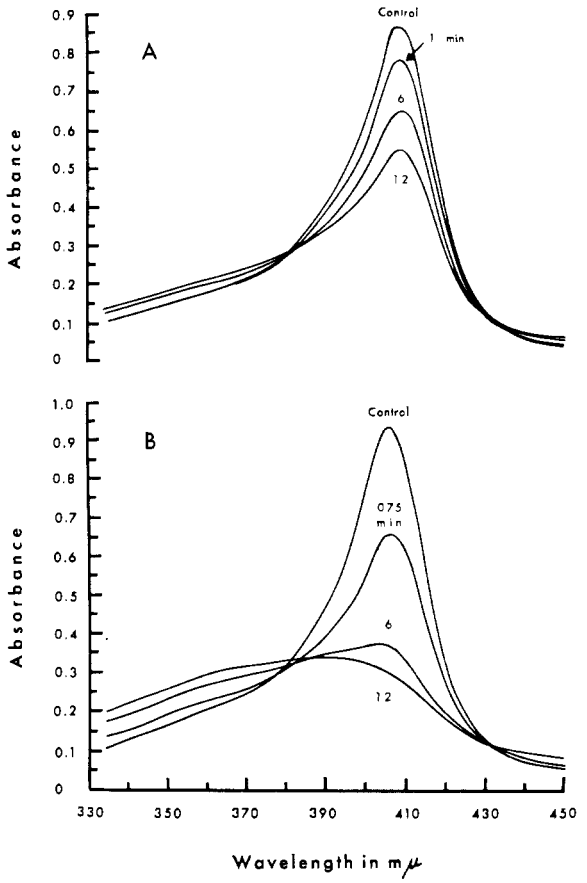


FIG. 9.—Comparative rates of reaction of ferrimyoglobin (A) and ferrihemoglobin (B) with 9×10^{-3} M $ZnAc_2$ at ionic strength 0.019 M and *p*H 6.4. In control, $ZnAc_2$ replaced by NaAc; 0.01% protein incubated at 36° for times shown.

oxidation-reduction potentials (Clark, 1960) reacts much faster than ferrimyoglobin (Fig. 9). On the other hand, carbonmonoxymyoglobin and carbonmonoxyhemoglobin, which have covalent iron-ligand bonds, react much more slowly than their corresponding ferri- compounds in which the bonds are ionic.[6] These observations of themselves focus attention upon the structural complex involving the heme and adjacent portions of the protein moiety as the site of attack of Zn.

Last, it is supposed that after rupture of the iron-imidazole linkage the ferriheme is still attached to the now conformationally altered protein via the hydrogen bonds between its propionic acid groups and side chains (one of which is the CD3 arginine residue) extending from the protein, which polymerizes rapidly and reversibly. The intermolecular forces responsible for the polymerization have not been delineated experimentally but, since acid-denatured myoglobin also tends to aggregate, several kinds of forces must be entertained, e.g., hydrogen bonds, Zn-bridges, and the aggregating tendency of hemin which may well be exposed on the surface of monomeric Zn-reacted myoglobin. In any event, interaction between the ferriheme moieties of the polymer is held responsible for suppression and broadening of the Soret band.

[6] The order of decreasing reactivity toward Zn of the different myoglobin and hemoglobin derivations is ferri- > ferro- > carbonmonoxy-. The greater reactivity of ferri- as compared to ferro- is probably related to labilization of ligand structure in the former by charge transfer to the iron. These experiments will be considered in detail elsewhere.

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