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A Dissociative Mechanism for the Dithionite Reduction of Cobalt(III) Myoglobin

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The kinetics of reduction to the divalent state of cobalt(III)–myoglobin (CoMb$^+$) by dithionite were studied under N_2 at 25 °C, $\mu = 0.5$. The reactions were biphasic and could be separated into two simultaneous first order reactions, both of which had the same dependence on dithionite concentration. At 2.5 mM $S_2O_4^{2-}$, the half-life of the first reaction was 1.4 minutes and the second 19 minutes. The reactions were pH independent between pH 6.9 and 7.8. The rate law was consistent with a dissociative mechanism, where an axial ligand dissociates from the cobalt sites before reduction by the SO_2 anion radical. Cobalt(II)–myoglobin was formed by reduction of CoMb$^+$ with isopropanol radicals, where this presumably rapid reduction occurs without the necessity of predissociation. After reoxidation of the CoMb$^+$ (which carries oxygen as CoMbO$_2$) and purification, the dithionite kinetics were again biphasic, with 40% of a fast component and 60% of a slower form. Dialysis techniques indicated that CoMb$^+$ had little rapid binding affinity for low concentrations of cyanide at pH 7.5. The CoMb$^+$ results are compared with the isoelectronic RuMb and RhMb$^+$ systems, all of which show internal hemichrome characteristics.

Introduction

Cobalt(II) substituted myoglobin (CoMb) and its hemoglobin analogue (CoHb) react reversibly with molecular oxygen [1], and the comparative chemistries [2–6] of the coboglobins, iron(II) based hemoglobins and mixed Co(II)/Fe(II) hybrids [1, 7] is an active research area. Relatively little is known about the cobalt(III) protein forms [1, 8, 9]. In contrast to methemoglobin (FeHb$^+$), CoMb$^+$ does not bind azide, fluoride, thiocyanate or pyridine, and reacts relatively slowly with cyanide [8]. When CoMb$^+$ was reduced to the cobaltous form by dithionite ($S_2O_4^{2-}$), this 'CoMb' was observed to carry dioxygen only after aging in a reduced condition for many hours [4], whereas oxygenation of the immediately reduced 'CoMb' was reported to simply reform the original CoMb$^+$ (or CoHb$^+$) [8]. To account for such properties, it was suggested [8] that CoMb$^+$ is a six coordinate 'internal hemichrome', with both the proximal and distal imidazole nitrogen atoms bonded to the cobalt(III) center.

We report the results of a study of the kinetics of reduction of CoMb$^+$ by dithionite. The suggested mechanism is compared to those found [10, 11] for FeMb$^+$ and has many features similar to the isoelectronic RuMb reaction with carbon monoxide [12], and the RhMb$^+$ reaction with cyanide.

Experimental

Preparation of ApoMb

A Sperm whale myoglobin (Sigma) solution was completely oxidized to metmyoglobin by the addition of solid potassium ferricyanide while monitoring the visible absorbance between 700 and 500 nm [13]. The excess ferricyanide was removed by gel filtration through a Sephadex G-25 column

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equilibrated with distilled water. The $FeMb^+$ solution was dialyzed against 0.01 *M* KH_2PO_4/1 m*M* EDTA pH 6, and then absorbed on a carboxymethylcellulose column equilibrated with the same buffer [14]. The main component, Band IV, was concentrated by ultrafiltration through a YM 10 membrane (Amicon).

The heme was removed by the acid/butanone method [15] and the apo protein was further prepared as previously described [16]. Excess butanone was removed by passing the apoMb solution over Sephadex G-25 equilibrated with 0.01 *M* KH_2PO_4/1 m*M* EDTA. The apoMb was then dialyzed overnight at 4 °C against 0.02 *M* KH_2PO_4/1 m*M* EDTA, pH 7.3, which was at 4 °C prior to dialysis. Under these conditions, no precipitation occurred. The apoMb concentration was determined by the extinction coefficient of 15.8×10^3 M^{-1} cm^{-1} at 280 nm [17]. After dialysis, the apoMb was placed in an ice bath and the pH was adjusted to 8.3 with cold 1 *M* Tris buffer, pH 8.3. To this solution, enough pyridine was added such that the pyridine concentration was 0.3%.

Preparation of CoMb⁺

A 20% excess of cobalt(III) protoporphyrin-IX was dissolved in a minimum amount of pyridine and diluted with deionized water to one-fifth the volume of the apoMb solution, and brought to 0 °C. This solution was added dropwise to the apoMb at 0 °C, and stirred slowly at 4 °C overnight. The $CoMb^+$ was then dialyzed against 0.01 *M* Tris pH 8.3 and passed over a DEAE cellulose column equilibrated with 0.05 *M* Tris pH 8.3 to remove excess cobalt porphyrin. Further purification was achieved by dialyzing the $CoMb^+$ solution against 0.01 *M* KH_2PO_4/1 m*M* EDTA pH 6 and absorbing the solution on a CMC cellulose column equilibrated with the same buffer. The major $CoMb^+$ component was eluted by washing the column with several column volumes of 0.01 *M* KH_2PO_4/1 m*M* EDTA pH 6.4 preceded by washing with the same buffer which contained 0.02 *M* NaCl, and finally with a buffer containing 0.04 *M* NaCl. The purified $CoMb^+$ was dialyzed against 0.05 *M* KH_2PO_4, pH 7.3, concentrated by ultrafiltration and stored below −15 °C. The purity of $CoMb^+$ was checked by isoelectric focusing using a 5% polyacrylamide gel with Ampholines (LKB) of 3.5 to 10 and 7–9 on a LKB Multiphor isoelectric focusing unit. Approximately 98% of the $CoMb^+$ moved as a single band, with an R_f identical to native $FeMb^+$.

Radiolysis

Steady state radiolysis was carried out with a Gammacell 220 ^{60}Co source with a dose rate of 3.2 krad/min. Optical absorption spectra before and after irradiation were measured in a Cary 219 spectrophotometer. The $CoMb^+$ solution for radiolysis (pH 7.3, 0.05 *M* KH_2PO_4) contained 2.5% isopropanol and was deoxygenated by bubbling with N_2O, making $(CH_3)_2COH$ the reducing species [18a].

Cyanide Analysis

A Tecnicon AutoAnalyzer II dialysis apparatus was used for [18b] studying the cyanide binding interactions with the metalloporphyrins and metalloproteins at pH 7.5, 0.1 *M* KH_2PO_4.

Kinetic Studies

The dithionite reduction kinetics were run at 25 °C with N_2 deaerated solutions at an ionic strength of 0.5 (Na_2SO_4) using 10^{-2} *M* 4-(2-hydroxyethyl)-1-piperazine sulfonic acid as a buffer from pH 7 to 8. Sodium dithionite was analyzed by titration against ferricyanide [19]. The kinetics were followed in the Soret region on a Beckman Acta III recording spectrophotometer. Pseudo first order conditions were used with a greater than hundred fold excess of dithionite (*ca.* 10^{-3} *M*) to $CoMb^+$ (*ca.* 3×10^{-6} *M*). The observed rates were found to be independent of $CoMb^+$ concentration between 7×10^{-6} and 1×10^{-6} *M*, at a constant dithionite concentration.

Results

$CoMb^+$ was reduced at pH 7.3 with either dithionite or the $(CH_3)_2COH$ radicals produced by radiolysis of aqueous i-PrOH solutions. In both cases, the absorption spectral changes were the same, and typical results are shown in Fig. 1. Excellent isosbestic points were found during the course of reduc-

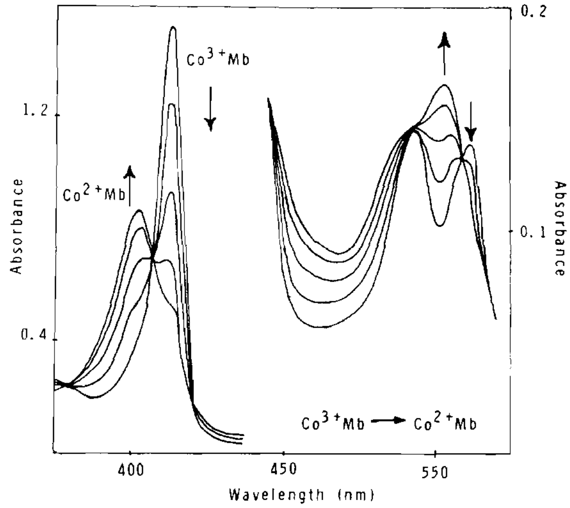


Fig. 1. Absorption spectral changes during the reduction of Cobalt(III) myoglobin by isopropanol radicals, pH 7.3, 0.05 *M* KH_2PO_4, 2.5% isopropanol, with N_2O. Above 400 nm, the same pattern is shown by dithionite.

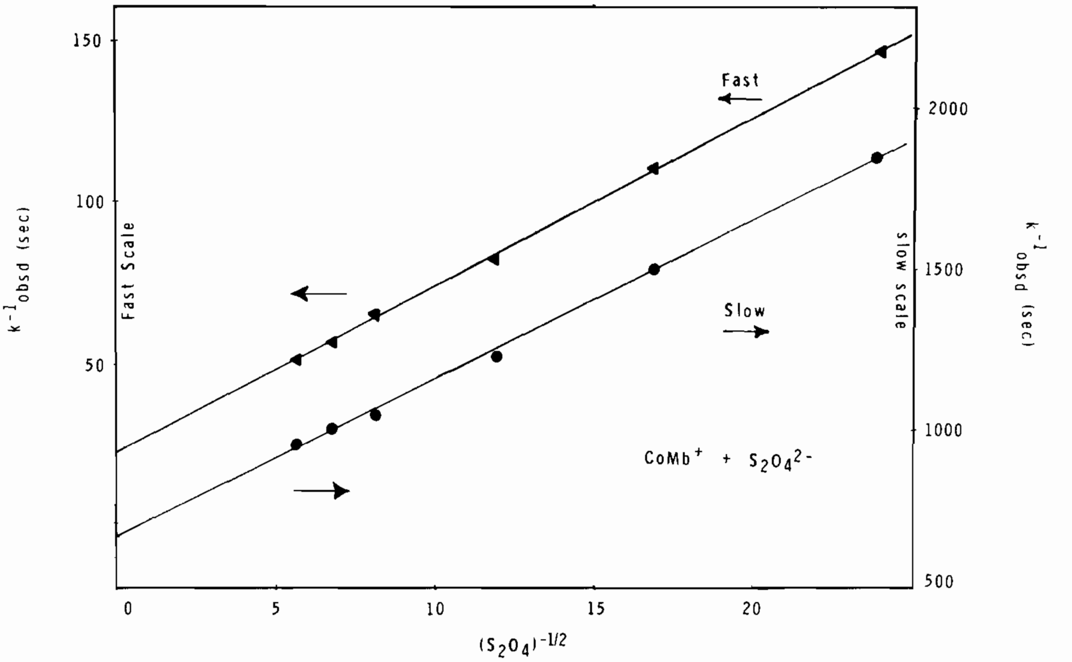


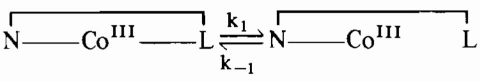
Fig. 2. Plots of $1/k_{obsd}$ *vs.* $1/(S_2O_4^{2-})^{1/2}$ for the $CoMb^+/S_2O_4^{2-}$ reactions.

tion at 357, 412, 492, 532, 566 and 590 nm. The product spectra had peak positions and relative intensities characteristic of CoMb [4]. Based on the known extinction coefficient of CoMb (λ_{max} = 406 nm, $\epsilon = 1.2 \times 10^5$ M^{-1} cm^{-1}), $CoMb^+$ had λ_{max} = 424 nm (1.9×10^5 M^{-1} cm^{-1}), 533 (1.4×10^4) and 571 (1.3×10^4). Similar patterns [8] were found for $CoHb^+$.

At pH 7.3, the $CoMb^+$ reduction kinetics were followed over a sixteen fold range in dithionite concentration (2.8×10^{-2} to 1.7×10^{-3} *M*). The reactions were biphasic, and could be analyzed into two simultaneous first order reactions [20]. For example, at 2.5×10^{-3} *M* $S_2O_4^{2-}$, the first reaction had a half-life of 1.4 minutes, while that for the second was *ca.* 19 minutes. Forty percent of the overall reaction was due to the fast component and sixty percent to the slower process. The rate constant, k_{obsd}, changed by less than 10% in the pH range 6.9 to 7.8, and several independent preparations of $CoMb^+$ gave the same results. The absorption spectra of $CoMb^+$ (and $CoHb^+$) [8] were independent of pH in the same range.

As shown in Fig. 2, plots of $1/k_{obsd}$ *vs.* $1/(S_2O_4^{2-})^{1/2}$ were linear for each reaction. The following mechanism is suggested:

$$S_2O_4^{2-} \underset{}{\overset{K_D}{\rightleftharpoons}} 2SO_2^- \qquad (1)$$



$$N—Co^{III}—L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} N—Co^{III} \quad L \qquad (2)$$

$$SO_2^- + N—Co^{III} \quad L \xrightarrow{k_2} N—Co^{II} \quad L + \text{Products} \qquad (3)$$

Step 1 is the rapid preequilibrium between $S_2O_4^{2-}$ and its radical anions SO_2^-, where [21] $K_D = 1.4 \times 10^{-9}$ *M*. With k_2 as the rate determining step and the five coordinate dissociation products of Step 2 as a steady state intermediate, the observed rate is of the form:

$$k_{obsd} = \frac{k_1 k_2 K_D^{1/2}(S_2O_4^{2-})^{1/2}}{k_{-1} + k_2 K_D^{1/2}(S_2O_4^{2-})^{1/2}} \qquad (4)$$

Upon rearrangement, $1/k_{obsd} = 1/k_1 + k_{-1}/(k_1 k_2 K_D^{1/2}(S_2O_4^{2-})^{1/2}$. For the faster component, $k_1 = 5 \times 10^{-2}$ s^{-1} and $k_{-1}/k_1 = 1.0 \times 10^{-5}$ *M*. For the slower reaction, $k_1 = 1.5 \times 10^{-3}$ s^{-1}, and $k_{-1}/k_1 = 2.8 \times 10^{-6}$ *M*.

After reduction, the CoMb solutions could be flushed with O_2 to destroy the excess $S_2O_4^{2-}$, and this product had spectra (λ_{max} = 423 nm, $\epsilon = 1.4 \times 10^5$ M^{-1} cm^{-1}) corresponding [4] to the oxygenated cobaltous myoglobin, $CoMbO_2$. Replacing the O_2 above the solutions with N_2 reformed the CoMb. This cycle could be repeated several times. Dithionite added to $CoMbO_2$ solutions rapidly reduces O_2 and produces CoMb.

At pH 7.5, (0.1 *M* KH_2PO_4), 1.5×10^{-4} *M* KCN was mixed with different concentrations of $CoMb^+$, $FeMb^+$, and the Co^{3+} and Fe^{3+} complexes of proto-

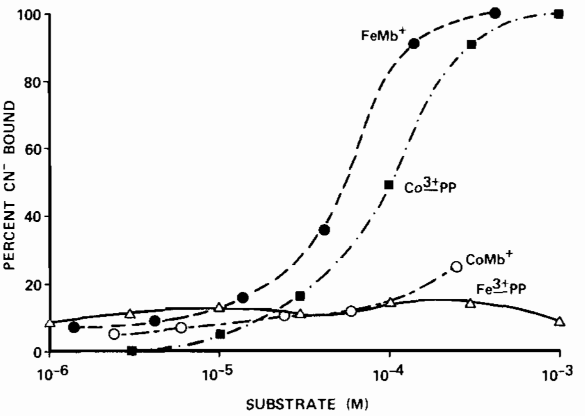


Fig. 3. Plot of percent bound cyanide *vs.* substrate concentration from dialysis experiments about two minutes after mixing metallo substrates at pH 7.5, 0.1 *M* KH_2PO_4 buffer with 1.5×10^{-4} *M* total KCN.

porphyrin-IX. A quantitative dialysis apparatus was used to measure the unbound cyanide concentrations within about two minutes of mixing. The cyanide binding profiles are shown in Fig. 3.

Discussion

Cobalt in the +I, +II and +III oxidation states can be stabilized by porphyrin molecules [22]. Reduction at pH 7.3 of $CoMb^+$ by both dithionite and radiolytically generated $(CH_3)_2\dot{C}OH$ radicals leads to the same CoMb product. Although the latter technique has been used to generate Co^I porphyrins [22], no such product was found to be produced from CoMb (in the absence of N_2O), which implies that if Co^IMb is produced, it perhaps reduces water or i-PrOH rapidly to reform the CoMb. Alternatively, it is possible that the radicals do not reduce the Co^{II} in CoMb, but rather react by different routes, *e.g.*, with the protein.

The fact that $CoMb^+$ (λ_{max} = 424 nm) and $CoMbO_2$ (λ_{max} = 423 nm) have about the same λ_{max} in the Soret (but different extinction coefficients) may lead to difficulties in assigning species present in solution. It was previously reported [4] that cobalt(III) porphyrins must be reduced to the +2 state before incorporation into apoMb to produce a CoMb which will carry O_2. Reconstitution of Co^{3+}–porphyrins into apoMb, followed by dithionite reduction (and removal) produces a 'CoMb' which cannot carry O_2, but reoxidizes in air to the $CoMb^+$ form. Only if this 'CoMb' was incubated with $S_2O_4^{2-}$ for 20 to 40 hours, would a normal oxygen carrying CoMb be produced upon dithionite removal. In our hands, however (and also noted by others [23]), CoMb formed by dithionite or radiolytic reduction of $CoMb^+$ can be cycled between the oxy and deoxy forms after the reductant is destroyed by O_2. Without such cycling experiments and knowledge of the relative extinction coefficients, $CoMbO_2$ is easily mistaken for $CoMb^+$. A qualitative distinction [23, 24] between the two forms arises in that $CoMbO_2$ is transformed by dithionite into CoMb much faster than is $CoMb^+$.

Changes in the absorption spectra as a function of ligand concentration are commonly used to probe hemoprotein complexation [13], and similar procedures are used for metalloporphyrins. However, cases are known for which complexation produces minimal shifts in absorption spectra [25]. Although the $CoHb^+$ spectra showed no alteration with a variety of anions and bases, 0.02 *M* cyanide (pH 9.2) produced changes in the CoHb α and β visible bands over a period of 24 hours, which were not accompanied by corresponding Soret shifts [8]. In related work, only kinetic evidence has been advanced for monocyano cobalt(III) porphyrins in solution [25]. Dialysis techniques do not depend on spectra, and the results of such a study on trivalent iron and cobalt porphyrins and myoglobins at pH 7.5 are shown in Fig. 3.

$FeMb^+$ and cobalt(III)–protoporphyrin-IX rapidly react with low concentrations of cyanide at pH 7.5 (where the ratio HCN/CN^- is 51:1). Fe(III)–protoporphyrin and $CoMb^+$ show much smaller effects under the same reaction conditions. The groups attached to the metals at this pH influence their reactivities. $FeMb^+$ is in the $Fe–OH_2$ form [13], while Co(III)–Proto probably exists [27] as a mixture of $Co–OH_2$ and $Co–OH^-$. Fe(III)–Proto may be predominantly the slowly reacting μ-oxo Fe–O–Fe dimer [28] at neutral pH's. The lack of rapid anation reactions of $CoHb^+$ have been ascribed to an internal hemichrome geometry, where both proximal F_8 and distal E_7 imidazole nitrogen atoms are bonded to the cobalt(III) axial positions [8]. Evidence for such coordination arises from EPR work of frozen $CoMb^+$ solutions, in which CoMb produced by radiolysis shows a pattern characteristic of a six coordinate CoMb. Upon warming, a five coordinate CoMb spectrum results [5].

The $CoMb^+/S_2O_4^{2-}$ data might also suggest an internal hemichrome structure. The biphasic kinetics indicate that $CoMb^+$ exists in two forms, which while having differing reactivities towards dithionite, show the same rate law dependence on dithionite concentration. The nature of these two forms is not clear; one is probably the internal bis-nitrogen bonded hemichrome, while the other may be a cobalt(III) center with a proximal imidazole and a distal water or pyridine group. As formulated in eqn. 1–3, SO_2^- cannot reduce the initially presumed six coordinate $CoMb^+$ reactants. Dissociation or rearrangement of ligands in the Co(III) coordination shell must occur before SO_2^- reduction. Isopropanol radicals are more powerful reductants than SO_2^-, and they reduce cobalt(III) porphyrins [29] and $FeMb^+$ and related heme proteins [30] in the range 10^8 to 10^9 M^{-1} s^{-1}. It would thus be expected that the $CoMb^+/(CH_3)_2\dot{C}OH$ reaction would occur faster than ligand dissociation in the $CoMb^+$ coordination sphere.

The $CoMb^+/S_2O_4^{2-}$ results are similar to work [10] on reduction of Fe^{3+}–Mb–X complexes, where SO_2^- reacts with only the dissociated $FeMb^+$ (FeMb–OH_2) species when X = F^-, N_3^-, CNO^-, SCN^- and HCO_3^-. An internal hemichrome $CoMb^+$ geometry might be expected to resemble Fe^{3+}–Mb–X* (X* = imidazole, pyridine or substituted forms of both) complexes in their reactivities. However, SO_2^- is found to react with the undissociated Fe^{3+}–Mb–X* adduct in these cases [10, 11].

A more profitable comparison can be drawn between the $CoMb^+/S_2O_4^{2-}$ system and the RuMb/CO, RuMb/O_2 and $RhMb^+/CN^-$ reactions [12], since Co^{3+}, Rh^{3+} and Ru^{2+} are isoelectronic d^6 metal ions. The reaction of RuMb with CO to produce the monocarbonyl adduct was also biphasic, where 65% of the sample reacted with CO about twenty times faster than the remaining *ca.* 35%, and a small amount of a third component was found to be unreactive. Similarly, the RuMb reaction with O_2 showed differing reactivities for all three components, with the final product being $RuMb^+$. In line with the $CoMb^+$ kinetics, a dissociative pathway was advanced for the RuMb/CO reaction, where several forms of the reactants exist. The presence of isosbestic points in both systems indicate that the differing forms of the reactants have fairly similar absorption spectra. The $RhMb^+$ was also postulated to be an internal hemichrome, due to its lack of reactivity with cyanide and related ligands [31].

Although porphyrin molecules tend to labilize coordinated metals [32], Ru^{2+} and Ru^{3+} are fairly substitution inert [33], and might be expected to retain their axial ligands in the RuMb/O_2 reaction, which was considered an outer sphere process [12]. Cobalt(III) is more substitution inert than Co^{2+}, and the distal ligand coordinated to Co^{3+} might be lost during the $CoMb^+$ reduction, forming a CoMb free to complex with dioxygen, as observed.

Assuming two initial $CoMb^+$ reactants and a single CoMb product, it was reasoned that if such a CoMb had no memory of its precursors, then reoxidation back to $CoMb^+$ might produce only one species, as evidenced by a single reactivity towards dithionite. However, after reoxidation with ferricyanide and the complete removal of this and other products by dialysis at 5 °C, which took 36 hours, the ensuing $CoMb^+/S_2O_4^{2-}$ reactions were again biphasic. Preliminary work indicates the existence of a single $CoMb^+$ (the fast reduced form) produced shortly after reoxidation of CoMb, which transforms with time into the two reactants initially studied. Efforts are underway to understand this process in more detail.

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References

1 B. M. Hoffman, in 'The Porphyrins', D. Dolphin, ed. Academic Press, New York 1979, Vol. VIII, Part B, Chap. 9, p. 403.

2 L. C. Dickinson and J. C. W. Chien, *J. Biol. Chem., 248,* 5005 (1973).

3 G. McLendon and P. Murphy, *J. Biol. Chem., 255,* 4035 (1980).

4 T. Yonetani, H. Yamamoto and G. Woodrow, *J. Biol. Chem., 249,* 682 (1974).

5 L. C. Dickson and M. C. R. Symons, *J. Phys. Chem., 86,* 917 (1982).

6 H. Yamamoto, F. J. Kane and T. Yonetani, *J. Biol. Chem., 249,* 691 (1974).

7 T. Inubushi and T. Yonetani, *Biochemistry, 22,* 1894 (1983).

8 S. Ridsdale, J. C. Cassat and J. Steinhardt, *J. Biol. Chem., 248,* 771 (1973).

9 H. Thiele, J. Behlke and W. Scheler, *Acta Biol. German., 12,* 19 (1964).

10 E. Olivas, D. J. A. de Waal and R. G. Wilkins, *J. Biol. Chem., 252,* 4038 (1977).

11 D. R. Eaton and R. G. Wilkins, *J. Biol. Chem., 253,* 908 (1978).

12 D. R. Paulson, A. W. Addison, D. Dolphin and B. R. James, *J. Biol. Chem., 254,* 7002 (1979).

13 E. Antonini and M. Brunori, 'Hemoglobin and Myoglobin in Their Reaction with Ligands', American Elsevier, New York.

14 K. D. Hardman, E. H. Eylar, D. Ray, L. Banaszak and F. R. N. Gurd, *J Biol. Chem., 241,* 432 (1966).

15 F. W. J. Teale, *Biochem. Biophys. Acta. 35,* 543 (1959).

16 F. K. Friedman, K. Alston and A. N. Schechter, *Anal. Biochem., 117,* 103 (1981).

17 L. Stryer, *J. Mol. Biology, 13,* 482 (1965).

18 (a) P. Neta, *Adv. Phys. Org. Chem., 72,* 233 (1976). (b) W. A. Groff, S. A. Cucinelli, P. Vicario and A. A. Kaminskis, *Clinical Toxicology, 11,* 159 (1977).

19 R. Pasternack, M. Cobb and N. Sutin, *Inorg. Chem., 14,* 866 (1975).

20 A. A. Frost and R. G. Pearson, 'Kinetics and Mechanisms', Wiley, New York, 1953, p. 162.

21 D. O. Lambeth and G. Palmer, *J. Biol. Chem., 248,* 6095 (1973).

22 S. Baral and P. Neta, *J. Phys. Chem., 87,* 1502 (1983).

23 B. M. Hoffman and D. H. Petering, *Proc. Natl. Acad. Sci., 67,* 637 (1970).

24 L. C. Dickinson, *J. Chem. Ed., 53,* 381 (1976).

25 F. A. Walker, E. Hui and J. M. Walker, *J. Am. Chem. Soc., 97,* 2390 (1975).

26 A. Adeyemo, A. Valiotti and P. Hambright, *Inorg. Chim. Acta Letters, 64,* L251 (1982).
27 R. Pasternack and M. A. Cobb, *J. Inorg. Nucl. Chem., 35,* 4327 (1973).
28 P. Hambright and P. B. Chock, *J. Inorg. Nucl. Chem., 37,* 2363 (1975).
29 P. Neta, *J. Phys. Chem., 85,* 3678 (1981).
30 J. Shieh, R. Cellars, M. Hoffman and I. Taub, 'Radiation Biology and Chemistry; Research Developments', H. Edwards, S. Nadaratnan, B. Parsons and G. Phillips, eds., Elsevier, New York, 1979, p. 179.
31 Y. Aoyama, K. Aoyagi, H. Toi and H. Ogoshi, *Inorg. Chem., 22,* 3046 (1983).
32 P. Hambright, in 'Porphyrins and Metalloporphyrins', K. M.Smith, ed., Elsevier, Amsterdam, 1975, Chap. 7.
33 P. C. Ford, *Coord. Chem. Reviews, 5,* 75 (1970).