Lee, Y. C. (1969) *Biochem. Biophys. Res. Commun. 35*, 161–167.
Lehmann, J., & Schröter, E. (1972) *Carbohydr. Res. 23*, 359–368.
Lehmann, J., & Ziegler, B. (1977) *Carbohydr. Res. 58*, 73–78.
Sinnott, M. L. (1978) *FEBS Lett. 94*, 1–8.
Sinnott, M. L., & Souchard, I. J. L. (1973) *Biochem. J. 133*, 89–98.
Sinnott, M. L., & Viratelle, O. M. (1973) *Biochem. J. 133*, 81–87.
Sinnott, M. L., Viratelle, O. M., & Withers, S. G. (1975) *Biochem. Soc. Trans. 3*, 1005–1009.
Sinnott, M. L., Withers, S. G., & Viratelle, O. M. (1978) *Biochem. J. 175*, 539–546.
Tenu, J.-P., Viratelle, O. M., Garnier, J., & Yon, J. M. (1971) *Eur. J. Biochem. 20*, 363–373.
Tenu, J.-P., Viratelle, O. M., & Yon, J. M. (1972) *Eur. J. Biochem. 26*, 112–118.
Viratelle, O. M., & Yon, J. M. (1973) *Eur. J. Biochem. 33*, 110–116.
Viratelle, O. M., Deschavanne, P. J., & Yon, J. M. (1975) 10th Meeting of FEBS, Abstract 606.
Wallenfels, K., & Weil, R. (1972) *Enzymes, 3rd Ed. 7*, 617–663.
Wentworth, D. F., & Wolfenden, R. (1974) *Biochemistry 13*, 4715–4720.
Withers, S. G., Jullien, M., Sinnott, M. L., Viratelle, O. M., & Yon, J. M. (1978) *Eur. J. Biochem. 87*, 249–256.

Role of Mixed Oxidation States in the Oxidation of Hemerythrin Species by Ferricyanide Ion†

Zdravko Bradić, Patricia C. Harrington, Ralph G. Wilkins,* and Gerald Yoneda

ABSTRACT: The oxidation of deoxy- and oxyhemerythrin to methemerythrin by $Fe(CN)_6^{3-}$ has been examined. The first step produces $(semi\text{-}met)_O$, a half-oxidized form of hemerythrin which has different spectral and kinetic properties from $(semi\text{-}met)_R$ which is produced by one-electron reduction of methemerythrin. Further oxidation of $(semi\text{-}met)_O$ to methemerythrin usually occurs only indirectly via disproportionation of $(semi\text{-}met)_O$ to met and deoxy forms within the octameric framework. Oxidation of $(semi\text{-}met)_R$ by $Fe(CN)_6^{3-}$ is a direct second-order reaction. $(Semi\text{-}met)_O$ is reduced rapidly by dithionite to deoxy, whereas that of $(semi\text{-}met)_R$ is a slow biphasic process. The oxidation of oxyhemerythrin occurs via the deoxy species, little, if any, reactivity being attributable to the oxy form. The oxidation of the azide adduct of $(semi\text{-}met)_R$ is biphasic, in which in step one N_3^- is removed during the oxidation to methemerythrin, which in the second step recombines with N_3^-. Rate parameters for all these processes at pH 8.2 and 6.3 for protein from *Phascolopsis gouldii* and *Themiste zostericola* have been obtained. The implications of these findings to hemerythrin chemistry are discussed.

Hemerythrin (Hr) occurs in the erythrocytes of certain marine worms in a polymeric, usually octameric, form. It is an interesting example of a respiratory protein which contains two linked nonheme irons in each subunit (Hendrickson, 1978; Kurtz et al., 1977; Loehr & Loehr, 1979; Stenkamp & Jensen, 1979). When both irons are in oxidation state +2, this (deoxy) form interacts reversibly with oxygen to give the oxy form. Both deoxy and oxy forms are easily oxidized [usually $Fe(CN)_6^{3-}$ is employed] to a met species containing irons only in the oxidation state +3. This is no longer O_2 sensitive but does react with a number of anions to form adducts with a wide range of stabilities (Keresztes-Nagy & Klotz, 1965; Meloon & Wilkins, 1976; Olivas et al., 1979).

Apart from its importance in preparative hemerythrin chemistry, a study of the oxidation of hemerythrin species to methemerythrin by $Fe(CN)_6^{3-}$ is of interest for several reasons. (a) The reaction of deoxy- and oxyhemerythrin with the iron(III)–cyanide complex is a noncomplementary redox reaction, involving a one-electron oxidant and a two-electron reductant. The role in the oxidation of the half-oxidized form of the protein which we shall term $(semi\text{-}met)_O$, in which one of the binuclear irons is +3 and the other is +2, can therefore be delineated. Furthermore, the results can be compared with those obtained by ferricyanide oxidation of a semi-met form which has been recently obtained by a one-electron reduction of the met form using dithionite (Harrington et al., 1978) and which we now term $(semi\text{-}met)_R$. (b) A comparison of the kinetic behavior of the deoxy and oxy forms will determine whether oxidation of the latter occurs directly or via the deoxy form with which it is in dissociative equilibrium. (c) Finally, the observation of further absorption changes following rapid oxidation of hemerythrin species is a distinct possibility since the geometries of, and even the nature of, the ligands attached to the iron sites in the iron(II) forms are likely to be quite different from those in the iron(III) species (Kurtz et al., 1977). Conformational adjustments will be necessary following the oxidation, and these might be reflected in spectral changes.

Apart from some interesting qualitative observations (Brunori et al., 1971), there have been no reported kinetics of oxidation of hemerythrin species by $Fe(CN)_6^{3-}$ and we describe a detailed study at pH 8.2 and 6.3 of this protein from two sipunculids, *Phascolopsis gouldii* and *Themiste zostericola*.[1] These are pH values for which we have previous data, and they are near the limits of the range of pH which can be conveniently studied with this protein.

† From the Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003. *Received March 12, 1980.* This work was supported by Grant HL 17828 from the National Institutes of Health, Division of Blood Diseases and Resources.

[1] This species obtained from Pacific Biomarine Supply was formerly considered to be *Themiste pyroides* and was so designated in previous publications by us and others (Gormley et al., 1978).

0006-2960/80/0419-4149$01.00/0

Materials and Methods

The marine worms *P. gouldii* and *T. zostericola* were obtained from Marine Biological Laboratory, Woods Hole, MA, and Pacific Biomarine Supply, Venice, CA, respectively. Oxyhemerythrin was obtained from the coelomic fluid of these worms (Klotz et al., 1957; Klipenstein et al., 1972) in which form it was stored in a freezer. The material from *T. zostericola* after ~6–8 weeks of storage gave poor kinetic traces for some of the reactions studied. Reprecipitation with $(NH_4)_2SO_4$ gave material which behaves the same as fresh material. Methemerythrin was prepared by dialyzing oxyhemerythrin against $Fe(CN)_6^{3-}$ and then several times against the appropriate buffer system. Deoxyhemerythrin was prepared (a) by overnight dialysis against dithionite and then several times against deaerated buffer and (b) by irradiation of a mixture of methemerythrin (0.1 mM), riboflavin (3–5 μM), and EDTA[2] (5 mM) for 30–35 min by light from a 300-W projector lamp some 20 cm from the solution. Method b was the more convenient but sometimes led at pH 6.3 to slightly turbid solutions. Materials from both preparations gave similar results. A change of concentration of riboflavin and EDTA was without effect on the kinetics of the processes studied. $(Semi\text{-}met)_R$ hemerythrin was prepared either (a) by anaerobic addition of dithionite ion to met (1.3 equiv of $S_2O_4^{2-}$/Fe–Fe unit) or (b) by irradiation of the mixture above for 90 s only. Attainment of the semi-met stage was conveniently monitored by the absorbance at 420 nm, where that of the met form is decreased by ~50%. For rapid kinetic studies, $(semi\text{-}met)_R$ was prepared by irradiating met in one of the driving syringes of a stopped-flow apparatus and was used within 1 or 2 min after preparation. This is necessary because of its tendency to disproportionate completely (*T. zostericola*) or partially (*P. gouldii*) relatively quickly [$t_{1/2} \simeq$ 5–7 min, 25 °C; Babcock et al. (1980)]. The azide adduct of either semi-met form was prepared by adding azide ion to semimethemerythrin in solution as soon as it had been prepared. The azide complex is stable toward disproportionation for hours provided O_2 is excluded (Babcock et al., 1980). For the study of the oxyhemerythrin reactions, small amounts of deoxyhemerythrin were treated with an aqueous oxygen solution of known concentration well in excess of the protein. Only a small correction need then be made for the loss of free O_2 by reaction with deoxyhemerythrin. We encountered no problems of denaturing of oxyhemerythrin when treated with $Fe(CN)_6^{3-}$ at 25 °C as mentioned by York & Beardon (1970). All other materials used were chemically pure. All preparations and manipulations of deoxy, semi-met, and ferricyanide solutions were with scrupulous exclusion of O_2. Cells with serum caps and gas-tight syringes were used in the spectral and kinetic work. A Beckman 24 recording spectrophotometer and a Gibson-Durrum stopped-flow apparatus were employed. Kinetic runs were carried out at 500 and 550 nm where $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ are both transparent and at 360 nm where the absorbance changes are larger. By use of a reactant concentration in excess of that of the protein, a first-order reaction or two successive first-order reactions were always observed, the semilog plots being linear for three to four half-lives. Protein concentrations used were 0.05–0.2 mM depending on absorbance changes. The concentrations of proteins are expressed on the basis of a single unit (molecular weight 13 500). The spectrum and kinetic properties at pH 8.2 of the immediate products of reaction of deoxy and $(semi\text{-}met)_R$ hemerythrin with $Fe(CN)_6^{3-}$ were obtained by using ~0.1 mM protein and 0.1 mM $Fe(CN)_6^{3-}$. After the first stage was complete (~2 and 1 min, respectively), either the spectrum was run (15 °C) or the solution was quickly added to one syringe of a stopped-flow apparatus and mixed with the reagent under investigation. Experiments at pH 6.3 used 0.05 M Mes, and at pH 8.2 0.05 M Tris was employed. Most runs were at 25 °C and I = 0.15 M, with added Na_2SO_4. All kinetic data are contained in supplementary tables (see paragraph at end of paper regarding supplementary material).

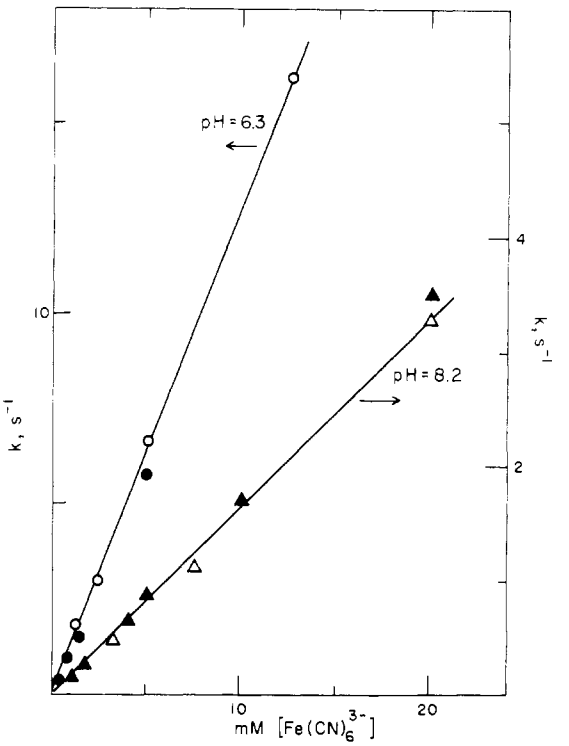

FIGURE 1: Observed first-order rate constant k vs. $[Fe(CN)_6^{3-}]$ for stage 1 of the reaction of deoxyhemerythrin (*P. gouldii*) with $Fe(CN)_6^{3-}$. (△) pH 8.0, I = 0.30 M; (▲) pH 8.0, I = 0.10 M; (○) pH 6.3, I = 0.45 M; (●) pH 6.3, I = 0.15 M. All were at 25 °C.

Results

Unless specified otherwise, the general behavior observed applies to protein from both species. The more extensive rate data were obtained with *P. gouldii* Hr, since some of the rates are slower and easier to measure accurately. For stoichiometry experiments and characterization of intermediates, there were often advantages in using *T. zostericola* Hr.

Reaction of Deoxyhemerythrin. Reaction of fully reduced hemerythrin with an excess of $Fe(CN)_6^{3-}$ shows, at pH 8.0–8.2 and 6.3, two stages which are easily separable. These reactions are best observed in the wavelength region 450–550 nm, where the faster phase is an absorbance increase, the slower phase is a decrease, and there is little absorbance by ferri- or ferrocyanide. Both reactions are nicely first order in absorbance change. *At pH 8.2*, the rate constant for stage 1 is directly proportional to the concentration of $Fe(CN)_6^{3-}$ (Figure 1) and that for stage 2 is independent of oxidant concentration over a wide range of concentration (Figure 2). The final spectrum, allowing for absorbances by other species [$Fe(CN)_6^{4-}$ and riboflavin] present, is similar to that of methemerythrin. Thus, the rate laws for stages 1 and 2 are respectively

$$\text{rate} = k[\text{Hr}] = k_1[\text{Hr}][Fe(CN)_6^{3-}] \quad (1)$$

$$\text{rate} = k[\text{Hr}'] = k_2[\text{Hr}'] \quad (2)$$

[2] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Mes, 4-morpholineethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

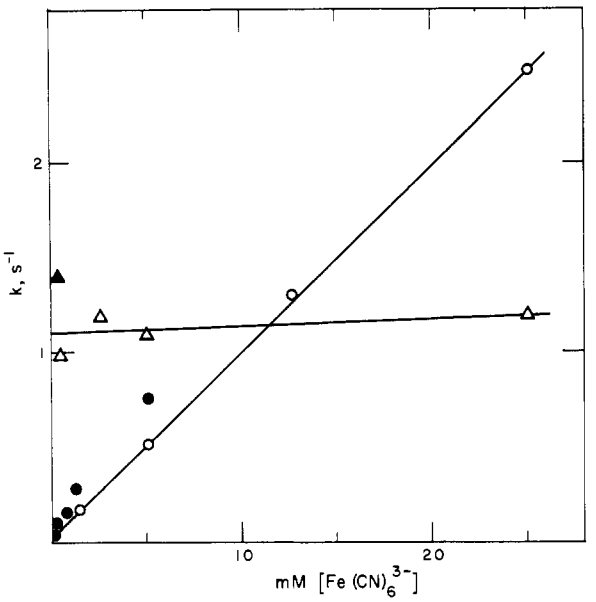

FIGURE 2: Observed first-order rate constant k vs. $[Fe(CN)_6^{3-}]$ for stage 2 of the reaction of deoxyhemerythrin (*P. gouldii*) with $Fe(CN)_6^{3-}$. (△) pH 8.0, I = 0.30 M; (▲) pH 8.0, I = 0.10 M; (○) pH 6.3, I = 0.45 M; (●) pH 6.3, I = 0.15 M. For pH 8.0, k must be multiplied by 10^{-3}. All were at 25 °C.

Table I: Rate Constants for Reactions of Hemerythrin Species with $Fe(CN)_6^{3-}$ at 25 °C and 0.15 M

species	pH	$10^{-3}k_1$ (M^{-1} s^{-1})	10^3k_2 (s^{-1})	10^3k_3 (s^{-1})
deoxy *P.g.*[a]	8.2	0.15	1.7	
deoxy *T.z.*[a]	8.2	100	1.2	2.6
deoxy *P.g.*	8.0[b]	0.17	1.1	
deoxy *P.g.*	6.3	1.2,[c] 0.17[c]		
deoxy *P.g.*	6.3	1.0,[b,c] 0.1[b,c]		
deoxy *T.z.*	6.3	>100	0.55	1.1
$(semi\text{-}met)_R$ *P.g.*	8.2	0.34		
$(semi\text{-}met)_R$ *T.z.*	8.2	400		

species	pH	k_7 (M^{-1} s^{-1})	k_8 (M^{-1} s^{-1})	k_{10} (M^{-1} s^{-1})
$(semi\text{-}met)_R$ *P.g.*	6.3[b]	26	8.8[d]	
N_3^- adduct *T.z.*	6.3[e]	1000		
met *P.g.*	8.2			1.7
met *P.g.*	6.3			12

[a] *P.g.* = *P. gouldii*; *T.z.* = *T. zostericola*. [b] I = 0.30 M. [c] Successive second-order rate constants. [d] At low $[N_3^-]$ (see Figure 6). [e] In presence of SCN^- in addition to N_3^-.

where Hr and Hr′ represent the reduced and intermediate hemerythrin species and k is the observed pseudo-first-order rate constant. The values of k_1 and k_2 at pH 8.2 for hemerythrin from both worms are shown in Table I. The values of k_1 and k_2 are little affected by a change of ionic strength from 0.02 to 0.5 M. Whereas k_1 is unaffected by the addition of 5 mM $NaClO_4$, k_2 is reduced by a factor of 2 compared with that in the absence of $NaClO_4$. The variation of k_2 over a temperature change from 15 to 30 °C gives $\Delta H^{\ddagger}_2$ = 15.2 kcal mol^{-1} and $\Delta S^{\ddagger}_2$ = −20 eu. The value of k_1, on the other hand, is virtually unchanged at temperatures 7, 14, and 25 °C leading to $\Delta H^{\ddagger}_1 \simeq 0$ kcal mol^{-1} and $\Delta S^{\ddagger}_1$ = −50 eu (*P. gouldii*).

By use of the slower reacting *P. gouldii* Hr and low concentrations of both protein and $Fe(CN)_6^{3-}$, the starting absorbance could be shown to be that of deoxyhemerythrin. Thus, there is no prior very rapid reaction within the mixing time. By use of the faster reacting *T. zostericola* Hr, thus

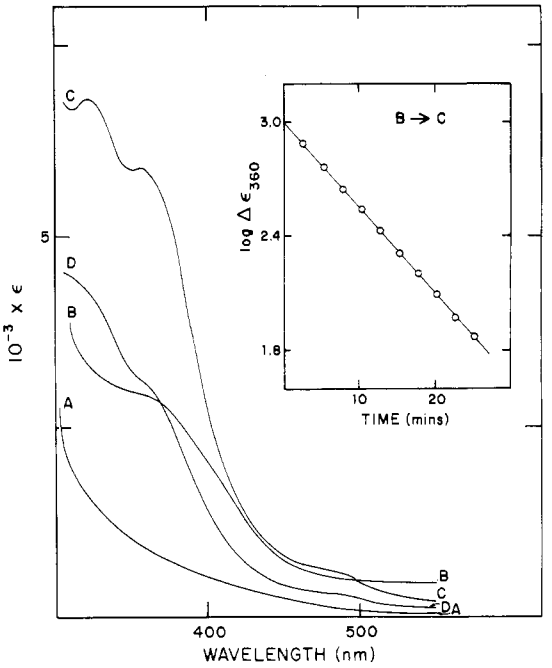

FIGURE 3: Spectra of deoxyhemerythrin (A); $(semi\text{-}met)_O$ obtained by using a mixture of 0.1 mM deoxyhemerythrin and 0.1 mM $Fe(CN)_6^{3-}$ 1–3 min after mixing (B); the final product, methemerythrin, from 0.1 mM deoxyhemerythrin and 0.2 mM $Fe(CN)_6^{3-}$ (C); $(semi\text{-}met)_R$ from reduction of methemerythrin (D). (Inset) Log $\Delta\epsilon_{360}$ vs. time for stage 2 of the reaction of deoxyhemerythrin with $Fe(CN)_6^{3-}$, B → C. All data were obtained at pH 8.2, I = 0.15 M, and T = 25 °C by using protein from *P. gouldii*.

speeding up stage 1 (Table I), it was possible to titrate deoxy with $Fe(CN)_6^{3-}$ and take readings quickly before stage 2 became important. The end of stage 1 occurred when one $Fe(CN)_6^{3-}$ had been added per 1 Fe(II)–Fe(II) unit. The spectrum of the product of this stage for *P. gouldii* Hr is shown as B of Figure 3. This species undergoes spectral changes even in the absence of $Fe(CN)_6^{3-}$. The process is better defined for *T. zostericola* Hr (see Discussion) and at λ = 360 and 550 nm:

$$\text{rate} = k_3[\text{Hr}'] \quad (3)$$

with values at 25 °C of k_3, $\Delta H^{\ddagger}_3$, and $\Delta S^{\ddagger}_3$ of 2.6×10^{-3} s^{-1}, 22.4 kcal mol^{-1}, and +6 eu, respectively. Addition of O_2 to B did not change the value of k_3 but leads to an equivalent amount of oxyhemerythrin and methemerythrin, whereas in the absence of O_2, equivalent amounts of deoxy- and methemerythrin result. The intermediate B was reduced (95 ± 5%) to deoxyhemerythrin *rapidly* by dithionite, and, by use of lowered temperatures and rapid manipulation of a solution containing B, the second-order rate constant for reaction with SO_2^- was determined as 4×10^5 M^{-1} s^{-1} (8 °C). The final product from the spontaneous change of B is reduced in a multiphasic fashion by $S_2O_4^{2-}$ over several hours, in a manner identical with that of methemerythrin (Harrington et al., 1978). In the presence of excess $Fe(CN)_6^{3-}$, B changes to C (Figure 3) which is the spectrum of methemerythrin, allowance having been made for the excess oxidant. In the conversion of B into C there are large absorbance increases at 360 nm, smaller decreases at 420 and 550 nm, and an isosbestic point at ~390 nm. Excellent first-order plots over four $t_{1/2}$ values are obtained at 360 nm (inset, Figure 3), and the rate constant is in excellent agreement with that obtained at 550 nm for stage 2.

At pH 6.3, the rate laws governing the reaction of *T. zostericola* deoxyhemerythrin with $Fe(CN)_6^{3-}$ are the same as

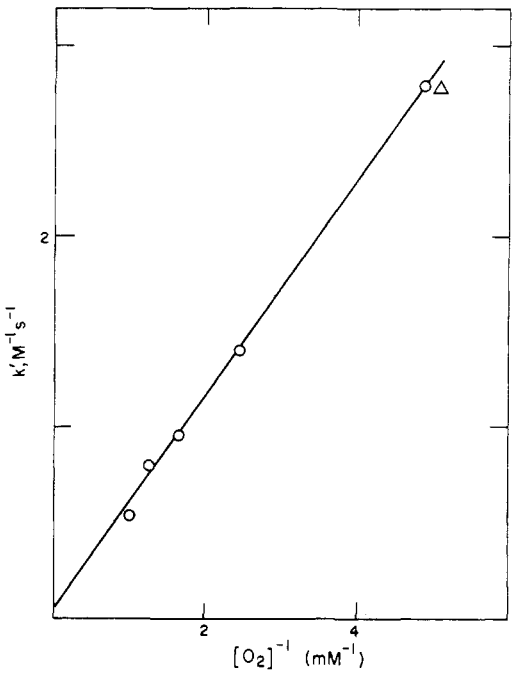

FIGURE 4: Second-order rate constant $k' = (k[Fe(CN)_6^{3-}]^{-1})$ vs. $[O_2]^{-1}$ for the first phase of the reaction of oxyhemerythrin (*P. gouldii*) with $Fe(CN)_6^{3-}$ at pH 8.2, I = 0.30 M, and 25 °C. (○) 3 mM $Fe(CN)_6^{3-}$; (△) 0.37 mM $Fe(CN)_6^{3-}$.

at pH 8.2. The same species is rapidly formed when 1 or >1 mol of $Fe(CN)_6^{3-}$ is added for each Fe_2 unit present. This species undergoes a slow reaction in the absence of $Fe(CN)_6^{3-}$ according to eq 3 and in the presence of $Fe(CN)_6^{3-}$ according to eq 2 to produce methemerythrin (Table I). With *P. gouldii* Hr, however, at pH 6.3, the rates of the two successive reactions observed are both $Fe(CN)_6^{3-}$ dependent (Figures 1 and 2), and the associated values of k_1 are shown in Table I. The immediate product of the two steps is *not the normal met form.* It has the same absorbance at 550 nm but is lower absorbing at lower wavelengths. It is reduced rapidly and quantitatively (≳90%) to deoxy by dithionite and reacts in a biphasic fashion with N_3^- to give an azide adduct spectrum quite unlike the azide complex of $(\text{semi-met})_R$ or met. Addition of 1 $Fe(CN)_6^{3-}$/deoxy species at pH 6.3 produces a spectrum quite similar to that obtained at pH 8.2. The rates of the processes studied were unaffected by the addition of $Fe(CN)_6^{4-}$, one of the products of the reactions.

Reaction of Oxyhemerythrin. There is a marked biphasic loss of absorbance at 500–550 nm when oxyhemerythrin is treated with $Fe(CN)_6^{3-}$ at pH 8.2, at which pH the detailed study was performed by using *P. gouldii* Hr. All the protein is converted finally into methemerythrin. If the concentrations of $Fe(CN)_6^{3-}$ and O_2 are in excess over that of (added) deoxyhemerythrin, then a first-order loss of oxyhemerythrin is observed in the faster stage. The dependence of the first-order rate constant k, associated with this stage, on the concentrations of $Fe(CN)_6^{3-}$ (0.5–12.0 mM) and free O_2 (0.20–1.0 mM) is given by (see Figure 4)

$$k = (a + b[O_2]^{-1})[Fe(CN)_6^{3-}] \qquad (4)$$

For the reaction scheme 5, the rate law 6 holds

$$\begin{array}{ccc} \text{deoxy} & \xrightarrow{k_1} & \text{product} \\ +O_2 \uparrow\downarrow K & & \\ \text{oxy} & \xrightarrow{k_4} & \text{product} \end{array} \qquad (5)$$

$$k = \left(\frac{k_1}{1 + K[O_2]} + \frac{k_4}{1 + (K[O_2])^{-1}} \right) [Fe(CN)_6^{3-}] \qquad (6)$$

where k_1 and k_4 are second-order rate constants for reaction of ferricyanide with deoxy- and oxyhemerythrin, respectively. The equilibrium between the deoxy and oxy forms is rapidly attained (deWaal & Wilkins, 1976). Since $K[O_2] > 1$, the observed eq 4 is obtained where $a = k_4$ and $b = k_1/K$. At pH 8.2, $k_4 \leq 0.1\ M^{-1}\ s^{-1}$ and by using a value of $K = 2 \times 10^5\ M^{-1}$, $k_1 = 116\ M^{-1}\ s^{-1}$. The value for k_1 is in good agreement with that determined in the experiments with deoxyhemerythrin as starting material (*P. gouldii*).

The second stage was studied at 550 and 360 nm (where the absorbance change is larger). With *P. gouldii* Hr, relatively large concentrations of $Fe(CN)_6^{3-}$ and small concentrations of O_2 were used in order to speed up the first process and ensure that it did not interfere with the slower phase. The first-order rate constants at pH 8.2 ($1.4 \times 10^{-3}\ s^{-1}$, *P. gouldii*, and $1.2 \times 10^{-3}\ s^{-1}$, *T. zostericola*) are independent of the concentrations of oxidant and O_2 and close in value to those associated with the second stage of the appropriate reaction of deoxyhemerythrin with $Fe(CN)_6^{3-}$. The absorbance changes are also similar. If oxyhemerythrin is treated with 1 mol equiv of $Fe(CN)_6^{3-}$ (*T. zostericola* was used to ensure that the first stage was rapid even with the low concentrations used), the product converts ($k = 2.3 \times 10^{-3}\ s^{-1}$) to a mixture of oxyhemerythrin and methemerythrin. The rate and product are identical with that observed when deoxyhemerythrin is treated with 1 mol equiv of $Fe(CN)_6^{3-}$ and the product reacted with O_2.

Less extensive rate studies using protein from *P. gouldii* at pH 6.3 and from *T. zostericola* at pH 8.2 show a similar pattern to that from *P. gouldii* at pH 8.2. Thus, (a) the addition of oxygen to the deoxy form slows down the oxidation by $Fe(CN)_6^{3-}$, (b) at constant O_2 concentration, the rate is first order in oxidant, (c) the value of k_1 extracted from the data is close to that obtained by using the deoxy form, while k_4, the rate constant for reaction of oxyhemerythrin, is always very small, and (d) the major absorbance loss of oxyhemerythrin is followed by a slower first-order process whose rate closely resembles the slower process observed when the starting reactant is deoxyhemerythrin.

Reaction of $(\text{Semi-met})_R$ Hemerythrin. The species $(\text{semi-met})_R$, produced by one-electron reduction of methemerythrin, reacts rapidly with $Fe(CN_6^{3-}$. Because it is difficult to obtain $(\text{semi-met})_R$ free of deoxy, which arises from some disproportionation of $(\text{semi-met})_R$ during manipulation, the kinetic traces sometimes show some slower changes with small absorbance amplitudes arising from the deoxy reaction. The major absorbance change reflects a single first-order reaction whose rate is linearly dependent on $[Fe(CN)_6^{3-}]$. The second-order rate constants k_1 for pH 8.2 are shown in Table I. The product at both pH values is methemerythrin as can be shown spectrally [by using stoichiometric amount of $Fe(CN)_6^{3-}$] and from its reaction rates with N_3^- and $S_2O_4^{2-}$, as well as from the spectrum of the azide adduct, which can be easily distinguished from that of the $(\text{semi-met})_R$–azide adduct (Harrington et al., 1978).

Azide ion forms a very stable adduct with $(\text{semi-met})_R$ at pH 6.3 and 8.2 (Harrington et al., 1978). Preliminary experiments indicated that the reaction of this semi-met–azide complex with $Fe(CN)_6^{3-}$ was best studied at pH 6.3. At 550 nm there is a decrease, followed by an increase, in absorbance. The final spectrum is quite close to that of methemerythrin

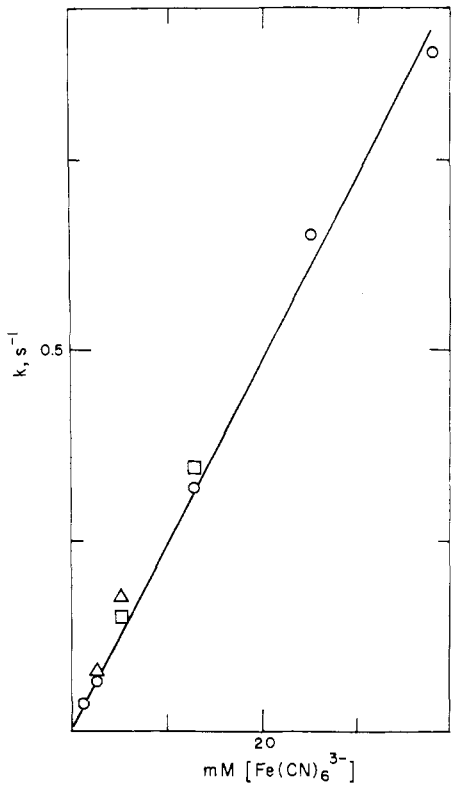

FIGURE 5: Observed first-order rate constant k vs. $[Fe(CN)_6^{3-}]$ for stage 1 of the reaction of $(semi\text{-}met)_R$ (*P. gouldii*)–azide adduct with $Fe(CN)_6^{3-}$. (○) 0.25 mM N_3^-; (△) 1.3 mM N_3^-; (□) 4.3 mM N_3^-. All were at pH 6.3, I = 0.45 M, and 25 °C.

azide. Both phases are pseudo first order with rate laws 7 and 8, respectively:

$$\text{rate} = k[\text{Hr}] = k_7[\text{Hr}][Fe(CN)_6^{3-}] \quad (7)$$

$$\text{rate} = k[\text{Hr}'] = k_8[\text{Hr}'][N_3^-] \quad (8)$$

Hr represents the initial reactant $(semi\text{-}met)_R{\cdot}N_3^-$ and Hr′ represents the intermediate (met). Plots of the observed rate constant for stage 1, k vs. $[Fe(CN)_6^{3-}]$ at various N_3^- concentrations, and for stage 2, k vs. $[N_3^-]$ at various $Fe(CN)_6^{3-}$ concentrations, are shown in Figures 5 and 6, respectively. The value of k_7 is independent of N_3^- from 0.25 to 4.3 mM, while k_8 does not vary when $Fe(CN)_6^{3-}$ is changed from 1.25 to 38 mM. At higher $[N_3^-]$, the $k/[N_3^-]$ plot deviates from linearity, as is observed in the studies of the anation of methemerythrin by N_3^- (Meloon & Wilkins, 1976). The values of k for the second stage and for the reaction of methemerythrin with N_3^- under the same conditions are quite close (Figure 6). Values of k_7 and k_8 are shown in Table I.

If the semi-met–azide adduct (*T. zostericola* was used for these experiments) is oxidized by $Fe(CN)_6^{3-}$ in the presence of low concentrations of SCN^- ion [which does not complex with the semi-met form (Harrington et al., 1978)], then two stages are again observed with first-order rate constants k given by eq 7 and 9:

$$\text{rate} = k[\text{Hr}'] = k_9[\text{Hr}'][SCN^-] \quad (9)$$

The final spectrum is that of the methemerythrin–thiocyante adduct. The value of k_9 at 10 mM SCN^- (32 M^{-1} s^{-1}) is close to that obtained (26 M^{-1} s^{-1}) for anation of methemerythrin by 10 mM SCN^- ion (Meloon & Wilkins, 1976).

Reaction of Methemerythrin. There is a very small absorbance decrease at 500–600 nm when 10 mM $Fe(CN)_6^{3-}$ is added to methemerythrin at pH 6.3 and 8.2. The rate of the decrease is first order in protein, and the associated rate

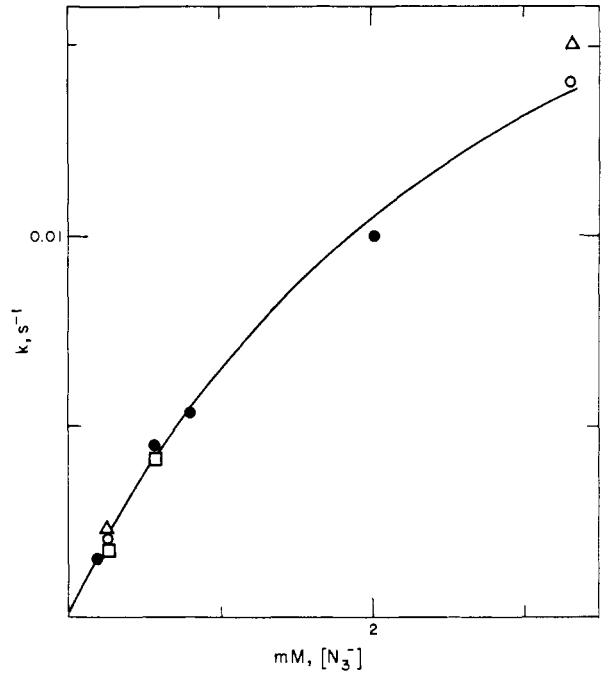

FIGURE 6: Observed first-order rate constant k vs. $[N_3^-]$ for stage 2 of the reaction of $(semi\text{-}met)_R$ (*P. gouldii*)–azide adduct with $Fe(CN)_6^{3-}$. (□) 1.1 mM $Fe(CN)_6^{3-}$, I = 0.15 M; (○) 2.5 mM $Fe(CN)_6^{3-}$, I = 0.45 M; (△) 38 mM $Fe(CN)_6^{3-}$, I = 0.45 M; (●) first-order rate constant for reaction of methemerythrin with N_3^- ion, I = 0.15 M. All were at pH 6.3 and 25 °C. The solid line is drawn through the experimental points and does not correspond to a specific rate law.

constant k varies with $Fe(CN)_6^{3-}$ concentration (in excess over that of protein) according to eq 10:

$$k = k_{-10} + k_{10}[Fe(CN)_6^{3-}] \quad (10)$$

This is consistent with an interaction:

$$\text{methemerythrin} + Fe(CN)_6^{3-} \underset{k_{-10}}{\overset{k_{10}}{\rightleftarrows}} \text{adduct} \quad (11)$$

Values of k_{10} at pH 6.3 and 8.2 are shown in Table I. It is difficult to obtain accurate values for k_{-10} for the intercept of the k vs. $[Fe(CN)_6^{3-}]$ plot but maximum values of 10^{-2} s^{-1} (pH 6.3) and 10^{-4} s^{-1} (pH 8.2) can be assessed. The values for the formation constant of the adduct (k_{10}/k_{-10}) are therefore $\geq 10^3$ and $\geq 10^4$ M^{-1}, respectively.

Discussion

The interaction of $Fe(CN)_6^{3-}$ with methemerythrin leads to slow and weak absorbance changes, so that this does not inferfere in observations of the reactions producing methemerythrin. Deoxyhemerythrin from both *P. gouldii* and *T. zostericola* is converted into methemerythrin by $Fe(CN)_6^{3-}$. The stoichiometry [$2Fe(CN)_6^{3-}$ for every Fe_2 unit] has been previously observed with deoxyhemerythrin from *Sipunculus nudus* (Brunori et al., 1971).

Spectral titration measurments using deoxyhemerythrin from *T. zostericola* show that 1 mol of $Fe(CN)_6^{3-}$ (per mol of Fe_2 unit) is rapidly consumed to give the $(semi\text{-}met)_O$ form and that the second mol of $Fe(CN)_6^{3-}$ converts the intermediate only slowly into methemerythrin (95 ± 5%). The second-order rate constant for the one-electron oxidation of deoxyhemerythrin (*P. gouldii*) by $Fe(CN)_6^{3-}$ (k_1, Table I) is much less than those for oxidation of the heme-containing proteins, horse heart cytochrome *c* (Morton et al., 1970; Creutz & Sutin, 1973; Cassatt & Marini, 1974), cytochrome c_2 (Wood & Cusanovich, 1975), and cytochrome *c*-551 (Antonini et al., 1970) but similar to that for oxidation of the iron–sulfur

protein HIPIP with $Fe(CN)_6^{3-}$, $\sim 10^3$ M^{-1} s^{-1} (Mizrahi et al., 1976; Rawlings et al., 1976). All these reactions, as well as that with deoxyhemerythrin (*P. gouldii*), are characterized by very small values for $\Delta H^{\ddagger}$, usually 0–3 kcal mol^{-1}, and large or very large negative $\Delta S^{\ddagger}$ values. The $\Delta H^{\ddagger}$ parameter, particularly, indicates that oxidation of these iron proteins by $Fe(CN)_6^{3-}$ is not a single step. The low $\Delta H^{\ddagger}$ values observed could be explained by evoking a labile preequilibrium with a negative ΔH° value, followed by a slower reaction with a normal $\Delta H^{\ddagger}$ value. The labile preequilibrium might involve weak $Fe(CN)_6^{3-}$ adduct formation within which electron transfer occurs. Alternatively, the normal form of deoxyhemerythrin might be in labile equilibrium with a more easily oxidized species. Quite surprisingly, the oxidation rate constant for deoxyhemerythrin from *T. zostericola* is 3 orders of magnitude larger than that from *P. gouldii*, and this is the largest rate difference we have so far observed for a variety of substitution and redox reactions. The $(semi\text{-}met)_R$ hemerythrin produced by one-electron reduction of methemerythrin is oxidized directly by $Fe(CN)_6^{3-}$, and the second-order rate constant is quite similar to that for the oxidation of deoxyhemerythrin from the same species and under similar conditions (Table I).

The product of the one-electron oxidation of deoxyhemerythrin, $(semi\text{-}met)_O$, has a distinctive spectrum different from that of $(semi\text{-}met)_R$ produced by the one-electron reduction of methemerythrin (Figure 3). It gives an EPR spectrum at liquid-helium temperature and reacts directly with dithionite ion but with O_2 and $Fe(CN)_6^{3-}$ (see below) only indirectly. The properties of these semi-met forms have been recently described (Babcock et al., 1980). Relevant to the present work is the spontaneous disproprotionation which the $(semi\text{-}met)_O$ form undergoes, in which there is a first-order redistribution of electrons within the octamer:

$$[Fe(II)Fe(III)]_8 \rightarrow [Fe(II)Fe(II)]_4[Fe(III)Fe(III)]_4 \quad (12)$$

This process is best studied by using *T. zostericola*, where the spontaneous change is ≥90% complete and nicely first order over several half-lives (k_3, Table I). With *P. gouldii* protein the disproportionation phenomenon appears more complex, with limited disproportionation occurring, as well as a $(semi\text{-}met)_O$–$(semi\text{-}met)_R$ interconversion (P. C. Harrington and R. G. Wilkins, unpublished experiments). The rate of the second stage in the oxidation of deoxyhemerythrin to methemerythrin is $[Fe(CN)_6^{3-}]$ independent (except for *P. gouldii* at pH 6.3). This second state is the conversion of $(semi\text{-}met)_O$ into met and is controlled by the disproportionation. The first-order rate constant for the second stage (k_2, Table I) is approximately half the value for the disproportionation (k_3) since one [Fe(II)Fe(III)] unit will be regenerated for every two lost by disproportionation. This relationship is similar to that for the disproportionation and dithionite reduction of $(semi\text{-}met)_R$ (Babcock et al., 1980). At pH 6.3, deoxyhemerythrin is rapidly oxidized to $(semi\text{-}met)_O$ and this disproportionates and reacts with $Fe(CN)_6^{3-}$ at about half the rate at pH 8.2, showing a very small effect of pH on disproportionation. The conversion of $(semi\text{-}met)_O$ into met by $Fe(CN)_6^{3-}$ at pH 8.2 also appears disproportionation controlled for *P. gouldii* Hr, with a rate constant quite close to that for *T. zostericola* Hr. However, at pH 6.3, the oxidation of $(semi\text{-}met)_O$ is a second-order process (Figure 2), and $Fe(CN)_6^{3-}$ can obviously oxidize directly. The product is interesting. It appears to be a fully oxidized hemerythrin species but has different spectral and kinetic properties from the usual methemerythrin. It appears to transform slowly to the usual met form, but observations of this are thwarted by the development of turbidity. The product of the reaction of $(semi\text{-}met)_R$ from *P. gouldii* with $Fe(CN)_6^{3-}$ at pH 6.3 is, however, the usual methemerythrin.

Our observations on the interaction of deoxyhemerythrin with $Fe(CN)_6^{3-}$ probably explain the results of Brunori et al. (1971). When deoxyhemerythrin (from *S. nudus*) was allowed to stand for "10 to 30 min" with incomplete amounts of $Fe(CN)_6^{3-}$, only fully reduced and fully oxidized protein was detected. It was concluded that oxidation occurs in an all or none fashion. In a sense this is correct, but any semi-met formed will probably have disproportionated in the standing time.

The remaining discussion is concerned with the effects of adduct formation by hemerythrin on its reactivity toward $Fe(CN)_6^{3-}$, specifically with the oxidation of oxyhemerythrin and the $(semi\text{-}met)_R$–azide adduct. The addition of oxygen to deoxyhemerythrin slows down the rate of oxidation by $Fe(CN)_6^{3-}$. Detailed studies of both species at pH 8.2 (and some rate observations at pH 6.3) show that the reaction proceeds substantially (≥95%) through the deoxy species. This is deduced from (a) the kinetic treatment and (b) the observation of the same $(semi\text{-}met)_O$ intermediate with the same properties as that observed in the reaction of deoxyhemerythrin with $Fe(CN)_6^{3-}$. Thus, oxyhemerythrin in the presence of excess $Fe(CN)_6^{3-}$ produces an intermediate which is converted into met at the disproportionation-controlled rate, and in the absence of $Fe(CN)_6^{3-}$ [by using 1 mol of $Fe(CN)_6^{3-}$/mol of oxyhemerythrin], it reacts with the oxygen present at a rate independent of the oxygen concentration and at the disproportionation rate. The product is *methemerythrin and oxyhemerythrin*, in equivalent concentrations. This latter observation is pertinent to previous literature reports. Thus, spectral titration of oxyhemerythrin with $Fe(CN)_6^{3-}$, leaving a long time after additions [8–12 h at 4 °C; York & Bearden (1970)], indicates a stoichiometry of $2Fe(CN)_6^{3-}$ for each oxyhemerythrin and a release of one O_2 (Boeri & Ghiretti-Magaldi, 1957; York & Beardon, 1970; Brunori et al., 1971). At lower than 2:1 ratios, an appropriate mixture of met- and oxyhemerythrin results. It is clear now that this simple stoichiometry results from a complex series of reactions in which oxy disssociates to deoxy, and the deoxy is rapidly oxidized to semi-met. This disproportionates slowly ($t_{1/2}$ = 75 min at 4 °C) to met and deoxy. The latter reacts with the $Fe(CN)_6^{3-}$ available and the remainder combines with the O_2 present to form oxyhemerythrin. The reactions of oxyhemerythrin with H_2O_2 (Bradić et al., 1979) and of oxymyoglobin with $Fe(CN)_6^{3-}$ (Antonini et al., 1965) and $FeEDTA^-$ (Cassatt et al., 1975) also proceed substantially through the deoxy form with little or no reactivity attributable to the oxy form. Part of this effect might be due to a smaller free energy change when oxyhemerythrin is the reactant. It does appear, however, from the limited number of systems so far reported that O_2 bonding, as well as being an integral feature of the respiratory process, may act to protect the protein against oxidation. It is also apparent that the deoxy form obtained rapidly (deWaal & Wilkins, 1976) by oxygen dissociation from oxyhemerythrin behaves the same toward $Fe(CN)_6^{3-}$ as deoxy obtained by lengthy reduction of methemerythrin.

In the oxidation at pH 6.3 of $(semi\text{-}met)_R$–azide adduct by $Fe(CN)_6^{3-}$, the rate law and spectral changes indicate that in the first step methemerythrin is produced *with the elimination of N_3^- ion*. The met produced then reacts with the excess N_3^- ion present to produce the met–azide adduct at the expected rate (Figure 6). An experiment which confirms this sequence is one in which semimethemerythrin in the presence of N_3^-

and SCN^- is oxidized by $Fe(CN)_6^{3-}$. Only azide ion binds to semi-met (Bradič et al., 1980) but the methemerythrin–thiocyanate adduct is the final product, since SCN^- reacts more rapidly than N_3^- with methemerythrin and would not replace azide from met–azide were this the product (Meloon & Wilkins, 1976). The elimination of N_3^- during the redox process suggests that $Fe(CN)_6^{3-}$ might bind at the anion site during the reaction, a complexity which is hinted at by the zero $\Delta H^{\ddagger}$ value for oxidation of deoxyhemerythrin (see above). The same general behavior is observed when the azide adduct of $(\text{semi-met})_O$ is treated with $Fe(CN)_6^{3-}$.

It is obvious that the binuclear iron unit in hemerythrin and the associated two-electron redox system allows a variety of redox reactions from within, and from outside, the octameric unit. These reactions are relevant to the stability and reactivity of the physiologically important deoxy- and oxyhemerythrin and merit further study.

Acknowledgments

We appreciate some measurements on the system which were made at an early stage of the study by Dr. R. Conrad.

Supplementary Material Available

Kinetic data for reactions of hemerythrin species with ferricyanide (5 pages). Ordering information is given on any current masthead page.

References

Antonini, E., Brunori, M. & Wyman, J. (1965) *Biochemistry 4*, 545.

Antonini, E., Finazzi-Agro, A., Guerrieri, P., Rotilio, G., & Mondovi, B. (1970) *J. Biol. Chem. 245*, 4847.

Babcock, L. M., Bradič, Z., Harrington, P. C., Wilkins, R. G., & Yoneda, G. S. (1980) *J. Am. Chem. Soc. 102*, 2849.

Boeri, E., & Ghiretti-Magaldi, A. (1957) *Biochim. Biophys. Acta 23*, 489.

Bradič, Z., Harrington, P. C., & Wilkins, R. G. (1979) in *Oxygen: Biochemical and Clinical Aspects* (Caughey, W. S., Ed.) p 557, Academic Press, New York.

Brunori, M., Rotilio, G. C., Rotilio, G., & Antonini, E. (1971) *FEBS Lett. 16*, 89.

Cassatt, J. C., & Marini, C. P. (1974) *Biochemistry 13*, 5323.

Cassatt, J. C., Marini, C. P., & Bender, J. W. (1975) *Biochemistry 14*, 5470.

Creutz, C., & Sutin, N. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1701.

deWaal, D. J. A., & Wilkins, R. G. (1976) *J. Biol. Chem. 251*, 2339.

Gormley, P. M., Loehr, J. S., Brimhall, B., & Hermodson, M. A. (1978) *Biochem. Biophys. Res. Commun. 85*, 1360.

Harrington, P. C., deWaal, D. J. A., & Wilkins, R. G. (1978) *Arch. Biochem. Biophys. 191*, 444.

Hendrickson, W. A. (1978) *Nav. Res. Rev. 31*, 1.

Keresztes-Nagy, S., & Klotz, I. M. (1965) *Biochemistry 4*, 919.

Klippenstein, G. L., Van Riper, D. A., & Oosterom, E. A. (1972) *J. Biol. Chem. 247*, 5959.

Klotz, I. M., Klotz, T. A., & Feiss, H. A. (1957) *Arch. Biochem. Biophys. 68*, 284.

Kurtz, D. M., Jr., Shriver, D. F., & Klotz, I. M. (1977) *Coord. Chem. Rev. 24*, 145.

Loehr, J. S., & Loehr, T. M. (1979) *Adv. Inorg. Biochem. 1*, 235.

Meloon, D. R., & Wilkins, R. G. (1976) *Biochemistry 15*, 1284.

Mizrahi, I. A., Wood, F. E., & Cusanovich, M. A. (1976) *Biochemistry 15*, 343.

Morton, R. A., Overnell, J., & Harbury, H. A. (1970) *J. Biol. Chem. 245*, 4653.

Olivas, E., deWaal, D. J. A., & Wilkins, R. G. (1979) *J. Inorg. Biochem. 11*, 205.

Rawlings, J., Wherland, S., & Gray, H. B. (1976) *J. Am. Chem. Soc. 98*, 2177.

Stenkamp, R. E., & Jensen, L. H. (1979) *Adv. Inorg. Biochem. 1*, 219.

Wood, F. E., & Cusanovich, M. (1975) *Bioinorg. Chem. 4*, 337.

York, J. L., & Bearden, A. J. (1970) *Biochemistry 9*, 4549.