Table II. Second-Order Rate Constants for the Reduction of Representative Di- and Trisubstituted Arenediazonium Salts ($ArN_2^+BF_4^-$) by Potassium Ferrocyanide and by Hemoglobin[a]

Ar	$k_X^{Fe(CN)_6^{4-}}$, M^{-1} s^{-1}	k_X^{Hb} (obsd), M^{-1} s^{-1}	k_X^{Hb} (calcd),[b] M^{-1} s^{-1}	k_X^{Hb} (obsd)/ k_X^{Hb} (calcd)
2-Cl-6-MeC_6H_3	24.0	194	145	1.34
2,4-$Me_2C_6H_3$	6.88	556	75.9	7.33
2,4,6-$Me_3C_6H_2$	4.07	19.8	56.2	0.35

[a] Same reaction conditions as described in Table I. [b] Calculated from the linear relationship of Figure 1: $\log k_X^{Hb} = 0.53(\log k_X^{Fe(CN)_6^{4-}}) + 1.43$.

from those predicted from ferrocyanide reductions (Table II). In the case of 2,4,6-trimethylbenzenediazonium tetrafluoroborate, steric factors apparently play a role in inhibiting electron transfer by hemoglobin. However, rate constants for both 2,4-dimethyl- and 2-chloro-6-methylbenzenediazonium tetrafluoroborate are higher than predicted if electron transfer had occurred without crossover onto the protein surface. One possible reason for the absence of σ-arylheme complexes in reactions of *o*-methylbenzenediazonium ions with hemoglobin is that the diazenyl radical undergoes intramolecular hydrogen abstraction from the *o*-methyl substituent.[37]

The formation of σ-bonded aryliron(III) adducts from reactions of hemoglobin with arenediazonium salts is limited relative to reactions of hemoglobin with arylhydrazines. In contrast to the corresponding hydrazine,[2] the *o*-methylbenzenediazonium ion does not form this adduct. In addition, *p*-nitrophenylhydrazine is effective in forming *N*-(*p*-nitrophenyl)protoporphyrin IX whereas the corresponding diazonium ion produces only the hydrogen abstraction product. The cause of these differences is apparently due to the existence of aryldiazene intermediates in reactions of hemoglobin with arylhydrazines.[15]

Acknowledgment. Support for this research from the National Institutes of Health (Grants ES 01673 and GM37469) is gratefully acknowledged. We thank Paul Saltman for his helpful discussions.

Registry No. $Fe(CN)_6^{4-}$, 13408-63-4; *p*-$NO_2C_6H_4N_2^+BF_4^-$, 456-27-9; *p*-$NCC_6H_4N_2^+BF_4^-$, 2252-32-6; *p*-$ClC_6H_4N_2^+BF_4^-$, 673-41-6; *p*-$FC_6H_4N_2^+BF_4^-$, 459-45-0; $C_6H_5N_2^+BF_4^-$, 369-57-3; *m*-$CH_3C_6H_4N_2^+BF_4^-$, 1422-76-0; *p*-$Me_2CHC_6H_4N_2^+BF_4^-$, 403-48-5; *p*-$CH_2COO^-C_6H_4N_2^+$, 110118-02-0; *p*-$CH_3C_6H_4N_2^+BF_4^-$, 459-44-9; *o*-$CH_3C_6H_4N_2^+BF_4^-$, 2093-46-1; *p*-(*n*-HexO)$C_6H_4N_2^+BF_4^-$, 88360-98-9; *p*-$CH_3OC_6H_4N_2^+BF_4^-$, 459-64-3; *p*-$PhNHC_6H_4N_2^+BF_4^-$, 2367-19-3; 2-Cl-6-$MeC_6H_4N_2^+BF_4^-$, 85070-46-8; 2,4-$Me_2C_6H_3N_2^+BF_4^-$, 452-02-8; 2,4,6-$Me_3C_6H_2N_2^+BF_4^-$, 23755-18-2.

(37) Doyle, M. P.; Siegfried, B.; Dellaria, J. F., Jr. *J. Org. Chem.* **1977**, *42*, 2426.

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Active-Site Chemistry of Hemerythrin: Mechanistic Routes in the Redox Interconversion of Deoxy and Met Forms

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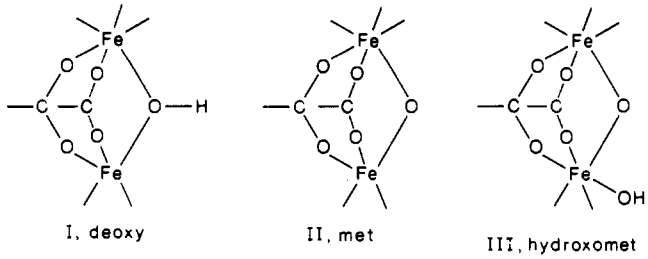
Two kinetic stages are observed in the oxidation of *Themiste zostericola* deoxyHr $Fe(II,II)_8$ to metHr $Fe(III,III)_8$ with $[Fe(CN)_6]^{3-}$, $[Co(dipic)_2]^-$, and $[Ru(NH_3)_5(CH_3CN)]^{3+}$ at 25 °C, pH 6.3–7.0 (Mes) and 7.4–9.0 (Tris), and I = 0.15 M (Na_2SO_4). With use of intensely colored spinach plastocyanin, PCu^{II}, as oxidant it has been shown that 1 equiv of oxidant is consumed in each stage. A feature of the reaction with $[Fe(CN)_6]^{3-}$ is the observation of saturation kinetics for the first stage at pH 6.3 (but not pH 8.2), consistent with association of $[Fe(CN)_6]^{3-}$ with deoxyHr (K = 4300 M^{-1}) prior to electron transfer. The second stage (k_2) is independent of the concentration and identity of oxidant, with rate constants 0.61 × 10^{-3} s^{-1} at pH 6.3 and 1.27 × 10^{-3} s^{-1} at pH 8.8. The product of this stage, corresponding to $(semi\text{-}met)_R$, reacts rapidly with excess oxidant to give metHr. With insufficient oxidant, however, the product disproportionates to a species (F) also in a rapid step. Studies on the reduction of the latter with $[Co(sep)]^{2+}$ are included. The reduction of metHr from *Phascolopsis gouldii* has also been investigated briefly and is shown to proceed in three stages by a route similar to that previously reported for *Themiste zostericola*.

Introduction

There have been significant advances recently in understanding the structure and properties of the binuclear Fe active site of hemerythrin, which appears to be the same in the monomer (myo) and octamer forms and to be independent of the source. From X-ray crystallography[1] and EXAFS,[2,3] structure I is indicated for the deoxy form, with histidine coordinated in the (five) terminal positions. The Fe(II)'s in I are only weakly antiferromagnetically coupled (−13 cm^{-1}),[4,5] which together with the evidence obtained from crystallography and EXAFS is consistent with a hydroxo-bridged structure. Strong antiferromagnetic coupling is however observed for the met form (J = −134 cm^{-1}), which indicates the presence of an oxo bridge. There are in addition μ-carboxylato ligands. Recent X-ray crystal studies[6] on metHr from *Themiste dyscritum* (pH <6.5) have indicated a structure in which one of the Fe(III) atoms is octahedral and the other trigonal bipyramidal as in II. It has been demonstrated that solvent OH^- coordinates to the five-coordinate Fe to give III in a relatively slow acid–base equilibrium ($t_{1/2} \approx$ 1 min at 25 °C),[7] the pK_a of which is ~7.8. At pH >9 resonance Raman studies have detected the hydroxomet form III.[8] X-ray crystallography,[6] EXAFS,[2,3] Mössbauer,[4] and



I, deoxy II, met III, hydroxomet

(1) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H.; McCallum, J.; Loehr, J. S. *Proc. Natl. Acad. Sci. (U.S.A.)* **1985**, *82*, 713.
(2) Hendrickson, W. A.; Co, M. S.; Smith, J. L.; Hodgson, K. O.; Klippenstein, G. L. *Proc. Natl. Acad. Sci. (U.S.A.)* **1982**, *79*, 6255.
(3) Elam, W. T.; Stern, E. A.; McCallum, J. D.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1982**, *104*, 6369; **1983**, *105*, 1919.
(4) Reem, R. C.; Solomon, E. I. *J. Am. Chem. Soc.* **1984**, *106*, 8323.
(5) Klotz, I. M.; Klippenstein, G. L.; Hendrickson, W. A. *Science (Washington, D.C.)* **1976**, *198*, 335.
(6) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. J. *J. Am. Chem. Soc.* **1984**, *106*, 618.
(7) Bradič, Z.; Wilkins, R. G. *Biochemistry* **1983**, *22*, 5396.
(8) McCallum, J. D.; Shiemke, A. K.; Loehr, J. S. *Biochemistry* **1983**, *23*, 2819.

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Raman studies[9] on the met form support a μ-oxo structure.

In a major contribution Wilkins and colleagues have identified the product of the one-electron oxidation of deoxyHr,[10,11] the $(\text{semi-met})_O$ form, and, in the one-electron reduction of metHr, the $(\text{semi-met})_R$ form.[12] Both have been characterized by EPR spectroscopy. The present work was undertaken to further establish stoichiometries and kinetic pathways for the oxidation of the deoxyHr octamer using different one-electron oxidants, and to relate these studies to those for the octamer metHr → deoxyHr change.[13,14]

As previously, pHs of 6.3 and 8.2 (or 8.8) were selected, which are on either side of the metHr active-site pK_a of 7.8.

We choose here to refer to the binuclear active site as e.g. Fe(II,II), which elsewhere is sometimes written Fe(II)Fe(II).

Experimental Section

Proteins. Octameric oxyHr was obtained from the coelomic fluid of the sipunculid worm *Themiste zostericola* (Pacific Bio-Marine, Venice, CA) by using a modified version of the procedure of Klippenstein et al.[15] Preparation and purification of metHr was achieved by dialysis of oxyHr against solutions of 10 mM $K_3[Fe(CN)_6]$ (BDH, AnalaR) at the required pH, followed by Sephadex G-100-120 gel-filtration chromatography. *Throughout this paper concentrations of protein are expressed as monomer subunits*; concentrations were established by using absorption measurements on metHr at 333 nm (ϵ 6500 M^{-1} cm^{-1} per monomer), which is an isosbestic point for the acid–base forms of metHr II and III.

To prepare deoxyHr the following procedure was adopted. A solution of pure metHr was concentrated by ultrafiltration (Amicon CF-25 cones) to a volume of ca. 1.5 mL (1×10^{-3} M). This was dialyzed against 0.05 M Tris/H_2SO_4 (300 mL) at pH 8.2, I = 0.15 M (Na_2SO_4), under N_2 for 6 h at 4 °C in a 500-mL flask. The dialysis medium was then replaced by the same buffer (300 mL) containing 1 g/L of sodium dithionite (BDH, GPR) at 20 °C. After 24 h the flask was attached to the apparatus as in Figure 1 at 4 °C, comprising a Sephadex G-100 gel-filtration column (1000 × 1.5 cm) equilibrated in buffer as for subsequent work, and with a further deoxygenated supply of buffer to deoxygenate the column. The deoxyHr solution was dialyzed against the same buffer for 36 h while the column was deoxygenated by passage of the deaerated buffer (ca. 3 L). The dialysis solution was removed and the dialysis bag pierced with a syringe needle, allowing protein to be loaded onto the column via suitable manipulation of stopcocks. Elution was carried out with buffer, yielding denatured protein (colorless) as the first fraction, followed by pure deoxyHr (pale yellow). Only the central portion (ca. 30%) was used for kinetics. The protein was collected and stored in a series of 20-mL glass bottles. DeoxyHr was determined as oxyHr at 500 nm (ϵ 2200 M^{-1} cm^{-1} per monomer).

Similar procedures were adopted for the preparation and purification of hemerythrin from *Phascolopsis gouldii*.

Plastocyanin was isolated from fresh spinach leaves by using the procedure of Ellefson et al. and purified as previously described.[16] Fully oxidized protein, PCu^{II}, was obtained by addition of a few crystals of $K_3[Fe(CN)_6]$. Excess oxidant was removed and deoxygenation achieved by dialysis (Sigma 250-7U bags) against deaerated buffer solutions (100-fold v/v excess; three changes) under N_2. Protein fractions having absorbance peak ratios $A_{278}/A_{587} < 1.4$ were used. Concentrations of PCu^{II} were determined by using an absorption coefficient of 4500 M^{-1} cm^{-1} at the 597-nm peak.[17]

Inorganic Complexes. These were prepared and purified to known UV–vis absorbance spectra peak positions, λ, nm (ϵ, M^{-1} cm^{-1}): ammonium bis(pyridine-2,6-dicarboxylato)cobaltate(III) hydrate, $NH_4[Co(dipic)_2]\cdot H_2O$, 510 (630);[18] (acetonitrile)pentaammineruthenium(III) perchlorate, $[Ru(NH_3)_5(CH_3CN)](ClO_4)_3$, 295 (575), 380 (138);[19] $[Co(sep)]Cl_3\cdot H_2O$, where sep denotes the sepulchrate cage ligand 1,3,6,8,10,13,16,19-octaazabicyclo[6.6.6]eicosane, 340 (16), 472 (109)[20] (converted to the sulfate salt as described);[13] potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$ (BDH, AnalaR), 420 (1010) (used without further purification). Solutions of $[Co(sep)]^{2+}$ were obtained by reduction of $[Co(sep)]^{3+}$ at a Hg-pool cathode, potential −0.5 V (vs. SCE), under N_2. Concentrations were determined by redox titration with $[Fe(phen)_3]^{3+}$ as oxidant (ϵ 5900 M^{-1} cm^{-1} at λ 540 nm).[21] A solution of $[Co(9\text{-}aneN_3)_2]^{2+}$, where 9-aneN$_3$ is 1,4,7-triazacyclononane, was prepared by electrolytic reduction of $[Co(9\text{-}aneN_3)_2]Cl_3\cdot H_2O$ (details as previously described).[13] Stock solutions of $[Ru(NH_3)_5(CH_3CN)]^{3+}$ were kept under Ar for periods of up to 20 min, after which base hydrolysis decomposition occurs.[21] Because decomposition of the complex is extensive at pH 8.2, no studies were possible at this pH. Other reagents, including the manganate(III) complex $K[Mn(cydta)]\cdot 2H_2O$, where cydta is 1,2-diaminocyclohexane-*N*,*N*,*N*',*N*'-tetraacetate, $K_4[Mo(CN)_8]\cdot 2H_2O$, $K_4[Zr(C_2O_4)_4]\cdot 5H_2O$, and $Ba_5[(CN)_5Fe(CN)Co(CN)_5]_2\cdot 6H_2O$, were as in other studies from this laboratory.[22,23]

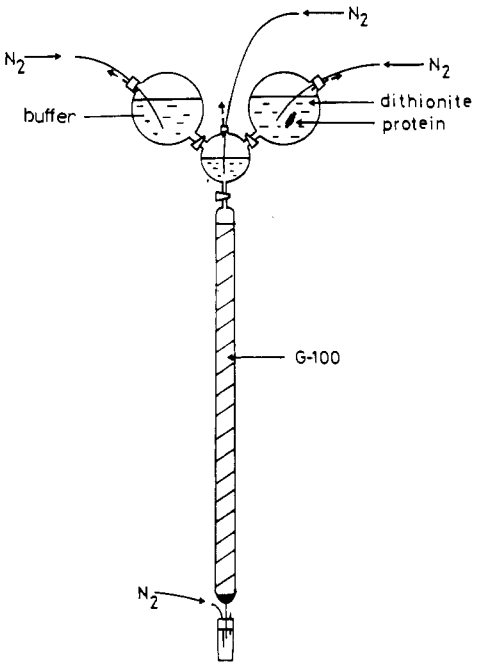


Figure 1. Apparatus used for the production and purification of deoxyhemerythrin.

Buffers and pH. Solutions of 0.05 M 2-morpholinoethanesulfonic acid (Mes, Sigma Chemicals) were adjusted to the required pH by addition of 1.0 M NaOH; the pH of 0.05 M tris(hydroxymethyl)aminomethane (Tris, Sigma Chemicals) was adjusted by the addition of 1.0 M H_2SO_4. Solutions over the pH range 6.3–7.0 (pK_a 6.15 for Mes at 25 °C), and 7.4–9.0 (pK_a 8.09 for Tris at 25 °C) were obtained.[24] All such solutions were adjusted to an ionic strength of 0.150 M with anhydrous Na_2SO_4. Measurements of pH were on a Radiometer (PHM 62) pH meter fitted with a Russell (CWR/322) combined Ag/AgCl reference/glass electrode. The meter was calibrated by using two standard buffer solutions (BDH, Colourkey) at pHs above and below the pH to be measured.

Kinetics. Rigorous air-free (N_2 or Ar) conditions were employed in all studies. Fast stages $k_{obsd} > 10^{-2}$ s^{-1}, were monitored on a Dionex D-110 stopped-flow spectrophotometer. Unless otherwise stated, inorganic reactants were maintained in large >10-fold excess of the protein.

(9) Freier, S. M.; Duff, L. L.; Shriver, D. F.; Klotz, I. M. *Arch. Biochem. Biophys.* **1980**, *205*, 449.
(10) Bradič, Z.; Harrington, P. C.; Wilkins, R. G.; Yoneda, G. *Biochemistry* **1980**, *19*, 4149.
(11) Harrington, P. C.; Wilkins, R. G. *J. Am. Chem. Soc.* **1981**, *103*, 1550.
(12) Muhoberac, B. B.; Wharton, D. C.; Babcock, L. M.; Harrington, P. C.; Wilkins, R. G. *Biochim. Biophys. Acta* **1980**, *626*, 337.
(13) Armstrong, G. D.; Ramasami, T.; Sykes, A. G. *Inorg. Chem.* **1985**, *24*, 3230.
(14) Armstrong, G. D.; Sykes, A. G. *Inorg. Chem.* **1986**, *25*, 3725.
(15) Klippenstein, G. L.; Von Riper, D. A.; Oosterom, E. A. *J. Biol. Chem.* **1972**, *247*, 5959.
(16) Ellefson, W. L.; Ulrich, E. A.; Krogman, D. W. *Methods Enzymol.* **1980**, *67*, 223. Sinclair-Day, J. D.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1986**, 2069.
(17) A plastocyanin PCu^{II} 597-nm peak (ϵ 4900 M^{-1} cm^{-1}) has been reported: Katah, S.; Shiratori, I.; Takamiya, S. *J. Biochem. (Tokyo)* **1962**, *51*, 32. The stoichiometries have to be adjusted downwards if this value is used.
(18) Mauk, A. G.; Coyle, C. L.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1979**, *101*, 5054.
(19) Clarke, R. E.; Ford, P. C. *Inorg. Chem.* **1970**, *9*, 227. Zenella, A. W.; Ford, P. C. *Inorg. Chem.* **1975**, *14*, 42.
(20) Creaser, I. I.; Geue, R. J.; Harrowfield, J. M.; Herlt, A. J.; Sargeson, A. M.; Snow, M. R.; Springborg, J. *J. Am. Chem. Soc.* **1982**, *104*, 6016.
(21) Schmidt, A. A.; Taylor, R. C. *J. Inorg. Nucl. Chem.* **1959**, *9*, 211.
(22) McGinnis, J.; Ingledew, W. J.; Sykes, A. G. *Inorg. Chem.* **1986**, *25*, 3730.
(23) Beoku-Betts, D.; Sykes, A. G. *Inorg. Chem.* **1985**, *24*, 1142.
(24) Smith, R. M.; Martell, A. E. In *Critical Stability Constants*; Plenum: New York, 1975; Vol. 2, p 20.
(25) E.g.: Sykes, A. G. *Chem. Soc. Rev.* **1985**, *14*, 283.

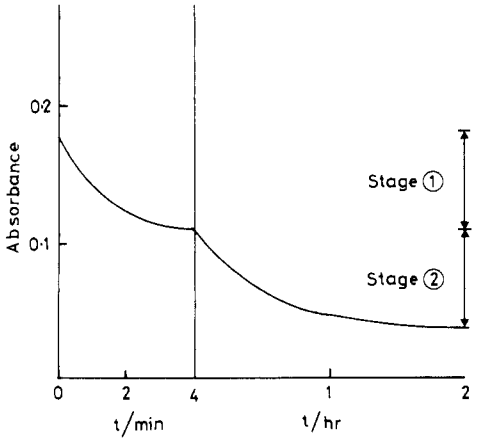


Figure 2. Stoichiometric experiment showing consumption of plastocyanin PCu^{II} (3.8×10^{-5} M) by *T.z.* deoxyHr octamer (1.4×10^{-5} M) at 597 nm (pH 6.3, I = 0.10 M (Na_2SO_4)).

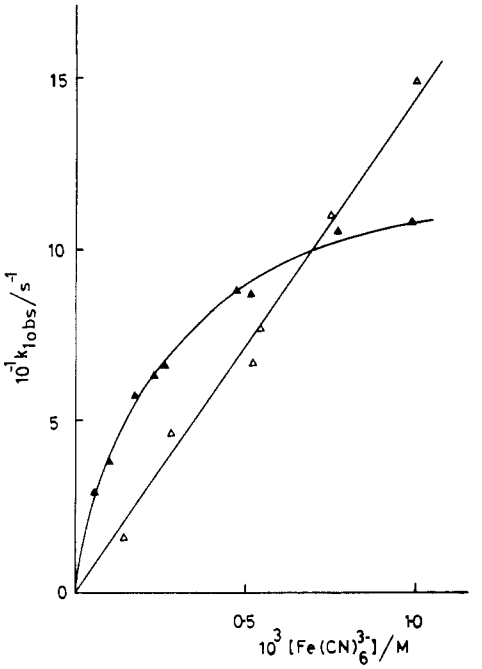


Figure 3. Dependence of $k_{1,obsd}$ (25 °C) for the first stage of oxidation of *T.z.* deoxyHr octamer (ca. 2×10^{-5} M) by $[Fe(CN)_6]^{3-}$ on oxidant concentration at pH 6.3 (▲) and pH 8.2 (△) (I = 0.15 M (Na_2SO_4)).

First-order rate constants were calculated from the slope of ln ($A_t - A_\infty$) vs. time plots, which were linear to at least 3 half-lives. Reactions were monitored at wavelengths in the range 320–400 nm depending on the reactant used. Slow stages were followed by using conventional UV–vis spectrophotometry on a Perkin-Elmer 554 instrument. All reactions were studied at 25.0 ± 0.1 °C, with I = 0.15 M (Na_2SO_4). The relatively higher errors for k_2 reflect difficulties in handling deoxyHr over long time intervals. As previously, the treatment is as if the protein were present as monomer units.

Analysis of Data. Unweighted linear and nonlinear least-squares programs were used as appropriate.

Results

Stoichiometry and Number of Stages. The blue Cu protein plastocyanin, PCu^{II} ((3.8–5.2) × 10^{-5} M), was used as oxidant for deoxyHr (*T.z.*) ((1.4–2.1) × 10^{-5} M). Absorbance changes were determined at the PCu^{II} peak at 597 nm, and calculations assumed $\Delta\epsilon$ = 4500 M^{-1} cm^{-1} for the one-electron reduction of PCu^{II} to colorless PCu^{I} (reduction potential 0.37 V).[17] Two stages were indicated (Figure 2). From three determinations carried out at pH 6.3 8 equiv (8.4 ± 0.6) of PCu^{II} was consumed per deoxyHr octamer in the first (rapid) stage, followed by consumption of a further 8 equiv (8.5 ± 0.6) in the second stage. No attempt was made to study the kinetics of the first stage. The kinetics of the second stage were monitored and indicated a uniphasic first-order decay process (see Table II).

Table I. Rate Constants $k_{1,obsd}$ for the First Stage of the Oxidation of *T.z.* Hr Octamer ($\sim 10^{-5}$ M) with $[Fe(CN)_6]^{3-}$ and $[Co(dipic)_2]^-$ at pH 6.3 (Mes) and 8.2 (Tris) (I = 0.15 M (Na_2SO_4))

oxidant	pH	10^3[oxidant], M	$k_{1,obsd}$, s^{-1}
$[Fe(CN)_6]^{3-}$	6.3	1.00	110
		0.77	107
		0.51	87
		0.48	88
		0.26	66
		0.23	63
		0.17	58
		0.10	38
		0.06[a]	29
	8.2	1.01	149
		0.76	111
		0.4	77
		0.51	67
		0.28	48
		0.16	16
$[Co(dipic)_2]^-$	6.3	1.72	0.50
		1.17	0.29
		0.92	0.24
		0.50	0.11
		0.34	0.10
		0.18	0.06
	8.3	2.27	0.30
		1.60	0.21
		1.10	0.16
		1.08	0.14
		0.89	0.11
		0.67	0.086
		0.39	0.051
		0.38	0.058
		0.17	0.033
		0.17	0.033
		0.07	0.016

[a] Complex in <10-fold excess. The rate constant was obtained from the initial slope of a ln ($A_t - A_\infty$) plot.

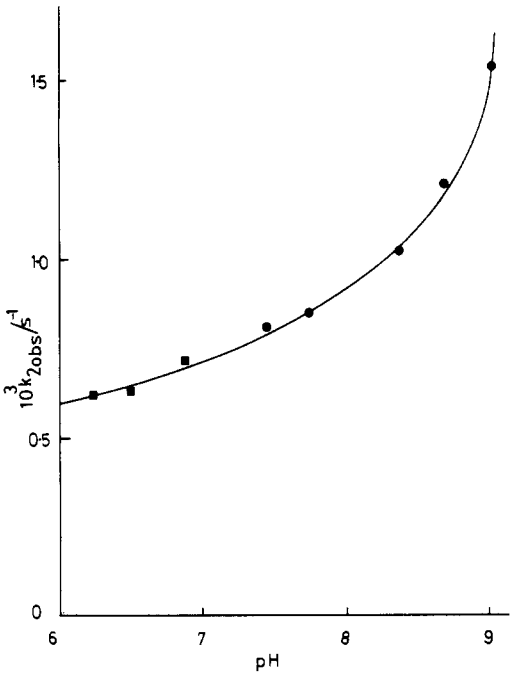


Figure 4. Effect of pH on rate constants $k_{2,obsd}$ (25 °C) for the second stage of the oxidation of *T.z.* deoxyHr octamer (5×10^{-5} M) by $[Co(dipic)_2]^-$ (6.7×10^{-4} M) in Mes (■) and Tris (●) buffers (I = 0.15 M (Na_2SO_4)).

First Stage of the Oxidation of deoxyHr (*T.z.*). First-order rate constants, k_{obsd}, for the oxidation of deoxyHr with $[Fe(CN)_6]^{3-}$

Table II. Rate Constants $k_{2,obsd}$ (25 °C) for the Second Stage of the Oxidation of *T.z.* deoxyHr Octamer ((5–1) × 10^{-5} M) with $[Fe(CN)_6]^{3-}$, $[Co(dipic)_2]^-$, $[Ru(NH_3)_5(CH_3CN)]^{3+}$, and PCu^{II} at pH 6.3 (Mes) and pH 8.8 (Tris) (I = 0.15 M (Na_2SO_4))

oxidant	pH	10^3[oxidant], M	$10^3 k_{2,obsd}$, s^{-1}
$[Fe(CN)_6]^{3-}$	6.3	1.00	0.72
		0.50	0.60
		0.25	0.62
	8.8	1.00	1.35
		0.50	1.35
$[Co(dipic)_2]^-$	6.3	1.00	0.68
		0.50	0.61
		0.25	0.61
	8.8	1.00	1.13
		0.25	1.25
$[Ru(NH_3)_5(CH_3CN)]^{3+}$	6.3	0.50	0.53
PCu^{II}	6.3	0.06	0.54[a]

[a] From PCu^{II} absorbance changes.

(Table I) at pH 8.2 give a linear dependence on $[Fe(CN)_6^{3-}]$ (eq 1), and k_1 = (1.5 ± 0.2) × 10^5 M^{-1} s^{-1}. At pH 6.3 however

$$k_{1,obsd} = k_1[Fe(CN)_6^{3-}] \quad (1)$$

saturation kinetic behavior is observed (Figure 3) consistent with the reaction scheme given by eq 2 and 3, from which eq 4 is obtained. A nonlinear least-squares fit gives K = 4300 ± 600

$$\text{deoxyHr} + [Fe(CN)_6]^{3-} \rightleftharpoons \text{deoxyHr,}[Fe(CN)_6]^{3-} \quad (2)$$

$$\text{deoxyHr,}[Fe(CN)_6]^{3-} \xrightarrow{k_{et}} \text{(semi-met)}_O + [Fe(CN)_6]^{4-} \quad (3)$$

$$k_{1,obsd} = \frac{Kk_{et}[Fe(CN)_6^{3-}]}{1 + K[Fe(CN)_6^{3-}]} \quad (4)$$

M^{-1} and k_{et} = 130 ± 6 s^{-1}, where the product (5.6 × 10^5 M^{-1} s^{-1}) is to be compared with k_1 = 1.5 × 10^5 M^{-1} s^{-1} at pH 8.2.

With $[Co(dipic)_2]^-$ as oxidant linear dependences of $k_{1,obsd}$ (Table I) on $[Co(dipic)_2^-]$ were obtained, as in (1), and gave second-order rate constants k_1 = 277 ± 20 M^{-1} s^{-1} (pH 6.3) and 135 ± 10 M^{-1} s^{-1} (pH 8.2).

The first stage was too rapid to monitor at pH 6.3 with $[Ru(NH_3)_5CH_3CN]^{3+}$ as oxidant, and absorbance changes were complete in 2 ms with deoxyHr (5 × 10^{-6} M) and $[Ru(NH_3)_5(CH_3CN)]^{3+}$ (5 × 10^{-5} M), indicating the second-order rate constant $k_1 > 5 \times 10^6$ M^{-1} s^{-1}. At pH 8.2 $[Ru(NH_3)_5(CH_3CN)]^{3+}$ was not sufficiently stable for kinetic studies.

Second Stage of the Oxidation of deoxyHr (*T.z.*). Rate constants $k_{2,obsd}$ (Table II) for the second stage showed no dependence on the identity or concentration of oxidant employed or on the concentration of deoxyHr (5-fold variation) and gave k_2 = (0.61 ± 0.07) × 10^{-3} s^{-1} at pH 6.3 and (1.27 ± 0.08) × 10^{-3} s^{-1} at pH 8.8. The trend observed is consistent with a more detailed study of the effect of pH on k_2 with $[Co(dipic)_2]^-$ as oxidant, which suggests a protein pK_a of >9. Conditions with pH >9 were difficult to study because of protein instability.

Properties of Intermediates (*T.z.*). UV–vis spectra were recorded for the $(semi\text{-}met)_O$ product (E) of the first stage of the oxidation of deoxyHr (1 × 10^{-4} M) with exactly 8 equiv (per octamer) of $[Ru(NH_3)_5(CH_3CN)]^{3+}$ or $[Fe(CN)_6]^{3-}$ at pH 6.3 (Figure 5). Similar experiments with the oxidant $[Co(dipic)_2]^-$ were less satisfactory because of the slowness of the initial redox step. A comparison of spectra of $(semi\text{-}met)_O$ (E) and $(semi\text{-}met)_R$ (B) from ref 13 (the same letter sequence is used as in the earlier work) is given in Figure 6. In the absence of any further oxidant $(semi\text{-}met)_O$ isomerizes to a species designated F with k_{obsd} = (0.60 ± 0.05) × 10^{-3} s^{-1}, which is identical with the k_2 value obtained in the presence of oxidant (Figure 5). On exposure of F to O_2, 4.1 ± 0.2 equiv of oxyHr is obtained (λ_{max} 500 nm, ϵ 2200 M^{-1} cm^{-1} per monomer),[26] indicative of four Fe(II,II) units and the formula $Fe(II,II)_4Fe(III,III)_4$.[11,27] Also on addition of N_3^- (3 × 10^{-3}M) changes in the UV–vis spectra were consistent with binding of 4.5 ± 0.5 mol of N_3^- per octamer to give azidometHr (λ_{max} 446 nm, ϵ 3.5 × 10^3 M^{-1} cm^{-1}).[28]

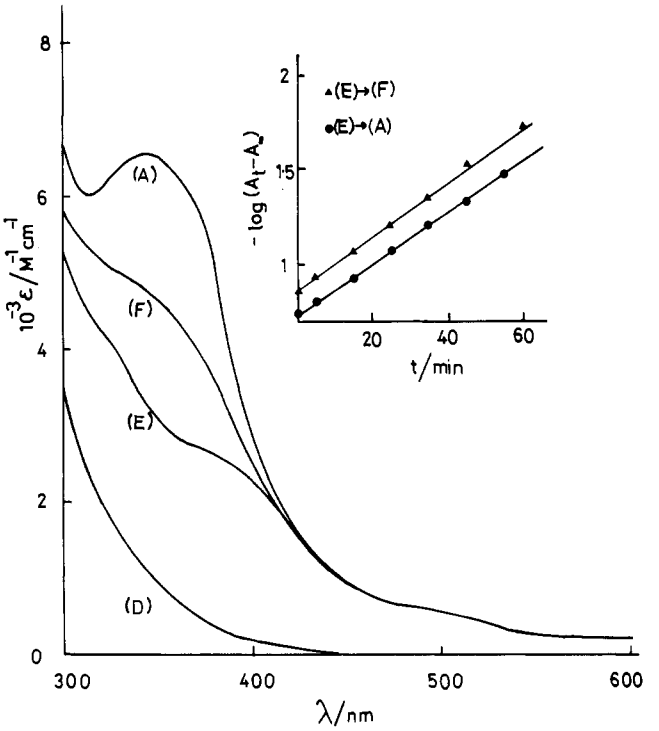


Figure 5. Spectra of octamer *T.z.* metHr (hydroxo form) (A), $(semi\text{-}met)_O$ (E), and deoxyHr (D). F is the product of isomerization of $(semi\text{-}met)_O$ in the absence of excess oxidant. The inset shows first-order plots for the conversions E–F and E–A (pH 6.3 (Tris), I = 0.15 M (Na_2SO_4)).

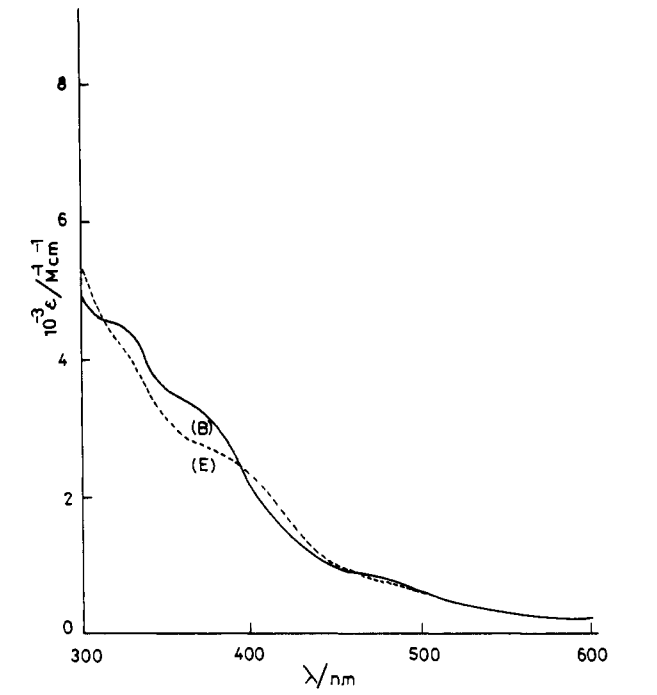


Figure 6. Spectra of *T.z.* hemerythrin octamer $(semi\text{-}met)_R$ (B, solid line) and $(semi\text{-}met)_O$ (E, broken line) (pH 6.3, I = 0.15 M (Na_2SO_4)).

(26) Garbett, K.; Darnall, D. W.; Klotz, I. M.; Williams, R. J. P. *Arch. Biochem. Biophys.* **1969**, *135*, 419.

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

(28) The reaction with N_3^- to give azidometHr does not appear to be confined to Fe(III,III) subunits and has been reported to occur also with semi-met forms.[11,27]

Table III. Rate Constants $k_{3,obsd}$ and $k_{4,obsd}$ (25 °C) for the Reduction of F[a] with $[Co(sep)]^{2+}$ at pH 8.2 (Tris) (I = 0.15 M (Na_2SO_4))

$10^3[Co(sep)^{2+}]$, M	$k_{3,obsd}$, s^{-1}	$10^3k_{4,obsd}$, s^{-1}	$10^3[Co(sep)^{2+}]$, M	$k_{3,obsd}$, s^{-1}	$10^3k_{4,obsd}$, s^{-1}
7.20[b]	1.54	4.0	0.75[b]	0.16	
3.25[b]	0.74		0.60[b]	0.10	
1.45[b]	0.28		0.30[c]		4.1
1.20[c]	0.24	4.2			

[a] Generated by addition of 8 equiv of $[Fe(CN)_6]^{3-}$ to *T.z.* deoxyHr (octamer). [b] [F] = 5×10^{-5} M. [c] [F] = 1×10^{-4} M.

The species designated F is also obtained on completion of the second stage of a metHr run with 8 equiv only of reductant.[13] The rate constant of 1.2×10^{-4} s^{-1} at pH 6.3 is the same as that obtained from the third stage in the presence of excess reductant.

Addition of exactly 16 equiv of $[Ru(NH_3)_5(CH_3CN)]^{3+}$ or $[Fe(CN)_6]^{3-}$ to deoxyHr (1×10^{-4} M) at pH 6.3 generated initially E, which then isomerizes ($k_2 = 0.60 \times 10^{-3}$ s^{-1}), followed by rapid oxidation to give metHr (A).

Similar processes are observed at pH 8.8 with $[Fe(CN)_6]^{3-}$ as oxidant. However, the spectrum of E displays anomalously high absorbance in the 500–600-nm region not apparent at pH 6.3. The absorbance is dependent on the concentration of $[Fe(CN)_6]^{3-}$ used and is attributed to some protein denaturation. Traces of Fe^{2+} are believed to be reacting with $[Fe(CN)_6]^{3-}$, giving a Prussian blue color. Rate constants for the convertion of E to F, and to A in the presence of excess $[Fe(CN)_6]^{3-}$, were unaffected. Oxidation of deoxyHr with a further oxidant, $[Mn(cydta)H_2O]^-$, at pH 8.8 gave the spectrum of E without contributions from the anomalous absorbance. This oxidant induces/competes with decomposition in the second stage, however, and no further studies were carried out.

Reduction of Intermediate F (*T.z.*) with $[Co(sep)]^{2+}$. Reduction of F, in this paper assigned the formula $Fe(II,II)_4Fe(III,III)_4$, to deoxyHr (D) occurs in two stages with $[Co(sep)]^{2+}$ as reductant at pH 8.2. The first stopped-flow stage gave rate constants $k_{3,obsd}$ (Table III), which displayed a linear dependence on $[Co(sep)^{2+}]$ (eq 5). The rate constant $k_3 = 200 \pm 10$ M^{-1} s^{-1} compares with

$$k_{3,obsd} = k_3[Co(sep)^{2+}] \quad (5)$$

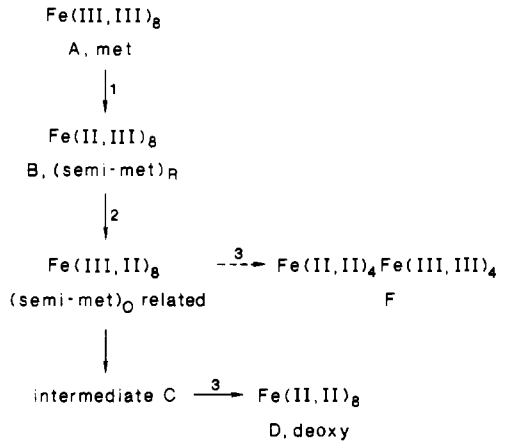
153 ± 6 M^{-1} s^{-1} from previous studies on the $[Co(sep)]^{2+}$ reduction of metHr.[13] The second stage occurs with the rate constant $k_{4,obsd}$ (Table III), which is independent of $[Co(sep)^{2+}]$, yielding $k_4 = (4.0 \pm 0.1) \times 10^{-3}$ s^{-1}. This is the same as observed previously for the second stage of the reaction of $[Co(sep)]^{2+}$ with metHr.

Reduction of metHr (*P.g.*). Three kinetic stages were observed in the $[Co(sep)]^{2+}$ and $[Co(9\text{-}aneN_3)_2]^{2+}$ reduction of metHr (*P.g.*) at pH 6.3 (reductant concentrations in the range $(0.5–2.0) \times 10^{-3}$ M). The second and third stages were independent of reductant. Rate constants obtained for $[Co(sep)]^{2+}$ (five runs) were $k_1 = 600 \pm 30$ M^{-1} s^{-1}, $k_2 = (2.3 \pm 0.03) \times 10^{-3}$ s^{-1}, and $k_3 = (0.8 \pm 0.04) \times 10^{-4}$ s^{-1}. Corresponding values for metHr (*T.z.*) are 260 M^{-1} s^{-1}, 2.0×10^{-3} s^{-1}, and 1.2×10^{-4} s^{-1}, respectively.[13] Rate constants for $[Co(9\text{-}aneN_3)_2]^{2+}$ reduction of metHr (*P.g.*) (four runs) are $k_1 = 38 \pm 1$ M^{-1} s^{-1} and $k_2 = (2.4 \pm 0.1) \times 10^{-3}$ s^{-1}, with precipitation making precise measurements of k_3 difficult. The corresponding value of k_1 for $[Co(9\text{-}aneN_3)_2]^{2+}$ reduction of metHr (*T.z.*) is 12.9 M^{-1} s^{-1}.[13] On reaction of the product of the second stage with O_2 more oxyHr (~6 mol) was detected as compared to the amount for the reaction of the corresponding *T.z.* product (~4 mol), suggesting some difference in behavior.

Discussion

As a result of studies by Wilkins and co-workers it has been established that the product of the first stage of reduction of metHr is $(semi\text{-}met)_R$ and that the corresponding product from the oxidation of deoxyHr is $(semi\text{-}met)_O$.[10–12] In the absence of further amounts of redox agent spontaneous disproportionation of the octamer semi-met forms occurs, i.e. $Fe(II,III)_8 \rightarrow Fe(II,II)_4Fe(III,III)_4$, and products containing Fe(II,II) (reaction with O_2) and Fe(III,III) (reaction with azide) binuclear subunits are obtained. If an excess of redox agent is present, the $(semi\text{-}met)_R$ and $(semi\text{-}met)_O$ forms are converted through to deoxy and met, respectively, at rates that are independent of the concentration of reductant and oxidant, respectively. It has been suggested that these processes proceed by disproportionation, which is rate-determining, and involve intramolecular electron transfer within the octamer over distances of 29–30 Å.[29]

Scheme I. Mechanism of Reduction of Octameric metHr (*T.z.* and *P.g.*), with Numbers Indicating the Different Kinetic Stages[a]



[a] The $(semi\text{-}met)_O$ related product of the second stage is possibly an incompletely formed $(semi\text{-}met)_O$ entity. Intermediate C approximates to a quarter-met form (*T.z.*). The broken line indicates a reaction occurring in the absence of sufficient reductant to proceed to C.

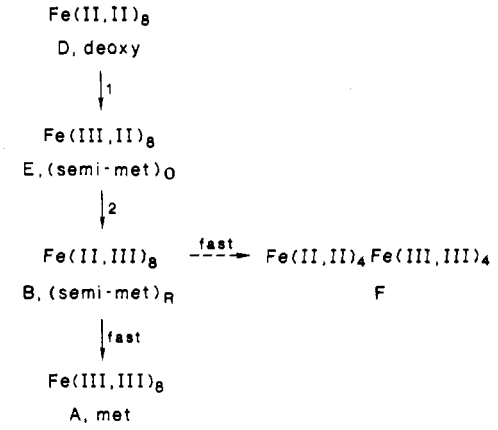
However, in a previous paper,[14] we have reported that with monomeric metHr (*T.z.*) the rate constants for the reductant-independent second stage are the same as those obtained for the reaction of the octamer at pH 8.2.[13] Since in the monomer case disproportionation can only occur by a bimolecular process, and the kinetics are first order, this suggests a mechanism in which intramolecular isomerization of Fe(II,III) to Fe(III,II) is the dominant process. With the monomer at pH 6.3 the interpretation is less clear-cut, since rate constants for the second stage exhibit a less than first-order dependence on reductant concentrations. Some other process(es) is contributing, including the possibility of binuclear disproportionation.

Armstrong et al.[30] have reported previously that in the absence of excess oxidant the equilibrium constant for $(semi\text{-}met)_O \rightleftharpoons (semi\text{-}met)_R$ is in the case of *T.z.* 0.05. The position of the equilibrium may be relevant to present considerations. The existence of six- and five-coordinate Fe's (I and II) makes reasonable the existence of nonidentical Fe(II,III) and Fe(III,II) forms. Maroney et al. have proposed from NMR contact shift measurements[31] that the six-coordinate and not the five-coordinate Fe of metHr appears to be reduced in the first stage, an observation in accordance with our earlier studies.[13] It would seem that because of its position in the protein, the six-coordinate Fe is more accessible to external electron-transfer reagents. This contrasts with the reactions of deoxyHr with O_2 and other (neutral) small reagents, which gain direct access to the five-coordinate Fe of the active site (see below).

The octamer metHr to deoxyHr conversion is considered first. Three kinetic stages have been reported for the reduction of $Fe(III,III)_8$ (*T.z.*) (Scheme I).[13] We report here three stages also in the reduction of $Fe(III,III)_8$ (*P.g.*) with $[Co(sep)]^{2+}$ and $[Co(9\text{-}aneN_3)_2]^{2+}$, in agreement with work of the Kurtz group using different reductants.[32] Whereas the kinetics of the first stage in which $(semi\text{-}met)_R$ is formed are dependent on the concentration (and identity) of the reductant, the second and third

(29) Harrington, P. C.; Wilkins, R. G. *Adv. Inorg. Biochem.* **1983**, *5*, 51.
(30) Armstrong, F. A.; Harrington, P. C.; Wilkins, R. G. *J. Inorg. Biochem.* **1985**, *18*, 83.
(31) Maroney, M. J.; Lauffer, R. B.; Que, L., Jr.; Kurtz, D. M. Jr *J. Am. Chem. Soc.* **1984**, *106*, 4951.
(32) Kurtz, D. M., Jr., personal communication.

Scheme II. Oxidation of Octameric deoxyHr (*T.z.*) at pH 6.3 and 8.2[a]



[a] The broken line represents a relatively fast reaction occurring in the absence of sufficient oxidant to proceed to metHr. The numbers indicate the different kinetic stages.

stages are independent of reductant. The absorbance change for the third stage is too large for it to correspond to the decay of the last Fe(III) of $Fe(II,II)_7Fe(II,III)$,[11,33] a point already addressed.[13] It seems clear therefore that the $(semi\text{-}met)_R \rightarrow$ deoxy conversion is difficult to achieve in a uniphasic process, and both isomerization *and* disproportionation may contribute.

We turn now to studies on the oxidation of $Fe(II,II)_8$. Two stages are observed. In the first the formation of $(semi\text{-}met)_O$ is dependent on the identity and concentration of oxidant, while the second is independent of both. For a given redox couple microscopic reversibility considerations lead us to expect processes similar (in reverse) to those observed for the met $\rightarrow$ deoxy conversion, in which case the $(semi\text{-}met)_O \rightarrow (semi\text{-}met)_R$ change is expected to contribute. Subsequent changes are then rapid. Spectra of intermediate states are not the same, however, since changes monitored are D $\rightarrow$ E $\rightarrow$ A, compared with A $\rightarrow$ B $\rightarrow$ C $\rightarrow$ D for the met to deoxy change,[13] and confirmation of reversibility is not immediately apparent. We have therefore examined the possibility that some sort of gated mechanism exists, whereby a slow change of, for example μ(OH) (for deoxy) to μ(O) (for met) might lead to a different route for the deoxy $\rightarrow$ met conversion. In recent work[34] it has been found that there is no D_2O effect either on the rate of reduction of met to $(semi\text{-}met)_R$ or on the rate of oxidation of $(semi\text{-}met)_R$ to met. A slow deprotonation as part of the deoxy $\rightarrow$ met change is not excluded.[35]

Scheme II is suggested for the deoxy $\rightarrow$ met (*T.z.*) interconversion. Relevant to this discussion, the rate constant for the second stage E $\rightarrow$ A is the same as that observed for the disproportionation E $\rightarrow$ F in the absence of excess oxidant (0.6×10^{-3} s^{-1} at pH 6.3; 1.3×10^{-3} s^{-1} at pH 8.8), indicating formation of the common intermediate $(semi\text{-}met)_R$. The same rate constant k_2 is observed whether 8 or 16 equiv of oxidant per octamer are added, and no statistical factor[29] appears to be required. The formula $Fe(II,II)_4Fe(III,III)_4$ proposed for F is consistent with its reaction with 4 mol of O_2 (to give oxyHr) and with 4 mol of N_3^- (to give azidomet). When F is reacted with $[Co(sep)]^{2+}$ at pH 8.2, two stages are observed, the first dependent on $[Co(sep)^{2+}]$ (200 M^{-1} s^{-1}) and the second independent of oxidant (4.0×10^{-3} s^{-1}). Rate constants are in satisfactory agreement with those obtained for the first two stages of the $[Co(sep)]^{2+}$ reduction of $Fe(III,III)_8$ (*T.z.*). In Scheme II, F could be positioned between B and A. Microscopic reversibility can be used as an argument against this, since for the reverse reaction A is believed to proceed directly to B.

The oxidation of $Fe(II,II)_8$ (*P.g.*) by $[Fe(CN)_6]^{3-}$ differs from that of *T.z.* in two respects.[10] First, the second stage is dependent on $[Fe(CN)_6^{3-}]$, which presumably occurs after rapid $(semi\text{-}met)_O \rightarrow (semi\text{-}met)_R$ isomerization, and second, a third stage is observed. The oxidation of monomeric Fe(II,II) (*T.z.*) by $[Fe(CN)_6]^{3-}$ exhibits similar features.[36] It has been suggested that the third stage, which occurs after $Fe(III,III)_8$ formation, corresponds to a conformational change.[10,36] Significantly, no $[H^+]$ dependence is reported for this process.

As far as rate constants (25 °C) for the first stage of the oxidation of deoxyHr (*T.z.*) are concerned ($[Co(dipic)_2]^-$ (135 M^{-1} s^{-1}) and $[Fe(CN)_6]^{3-}$ (1.5×10^5 M^{-1} s^{-1}) at pH 8.2), these reflect the self-exchange characteristics for $[Co(dipic)_2]^{-,2-}$ (1.0×10^{-5} M^{-1} s^{-1}) and $[Fe(CN)_6]^{3-,4-}$ (3.2×10^4 M^{-1} s^{-1}) rather than the reduction potentials, 0.75 and 0.41 V, respectively.[36,37] The poor self-exchange characteristics for Co(III)/Co(II) reactions have been noted previously and are related to high reorganization energy requirements.[39] A reduction potential of 0.31 V has been reported for the $(semi\text{-}met)_O$/deoxyHr couple.[30]

The variation of rate constants for the $[Co(sep)]^{2+}$ reduction of octameric metHr (*T.z.*) with pH gives a pK_a of 7.6,[13] and it is of interest whether this process is linked to the interconversion of active-site structures II and III ($pK_a \approx 7.8$).[7] We note however that pH 6.3 to pH 8.2 rate constant ratios for the oxidation of octameric deoxyHr with $[Fe(CN)_6]^{3-}$ (3.7) and $[Co(dipic)_2]^-$ (2.0) are similar to those observed for the reductions with $[Co(sep)]^{2+}$ (2.0) and $[Co(9\text{-}aneN_3)_2]^{2+}$ (1.6). There is no correlation with charge on the inorganic complex, and the ratios suggest that some residue or residues on the protein are responsible for the effect of pH observed.

The second-order rate constant for $[Fe(CN)_6]^{3-}$ at pH 6.3 of 5.6×10^5 M^{-1} s^{-1} is made up of association ($K = 4300$ M^{-1}) followed by electron transfer ($k_{et} = 130$ s^{-1}). With $[Co(dipic)_2]^-$ (at pH 6.3 and 8.2) and $[Fe(CN)_6]^{3-}$ (at pH 8.2) as oxidants saturation kinetics are not observed. It would appear that there is more extensive protonation of deoxyHr (*T.z.*) at pH 6.3, and favorable electrostatic association with $[Fe(CN)_6]^{3-}$ is observed. Studies by Bradič et al.[40] have demonstrated that $[Fe(CN)_6]^{4-}$ does not bind at the active site of deoxyHr and, more generally, only neutral reactants, including O_2 and H_2O_2 in redox processes,[41] and HN_3, HF, and HNCO (addition) appear to gain access to the five-coordinate Fe at the active site. Control is presumably determined by the nature of an access channel. Association of the oxidant $[Fe(CN)_6]^{3-}$ on the other hand is believed to occur at a site on the protein surface close to the six-coordinate Fe. Attempts in the present work to further establish this process by using the more highly charged redox-inactive complexes $[Mo(CN)_8]^{4-}$ and $[Zr(C_2O_4)_4]^{4-}$ at pH 6.3 resulted in protein denaturation. With the more highly charged $[(CN)_5FeCNCo(CN)_5]^{5-}$ as 1-equiv oxidant for deoxyHr at pH 6.3, the reaction was too fast for stopped-flow studies ($k_1 > 5 \times 10^6$ M^{-1} s^{-1}). The extensive association of $[Fe(CN)_6]^{3-}$, $K = 4300$ M^{-1} at pH 6.3, decreasing to a value of less than one-tenth of this at pH 8.2 (an estimate based on the observation that saturation kinetics are not observed) is an unusually large effect and is particularly so in view of the small overall charge on hemerythrin (*T.z.*) of around zero at pH $\sim$7 (p*I* 6.7).[42,43] The results obtained suggest that a positive patch

(33) Harrington, P. C.; DeWaal, D. J. A.; Wilkins, R. G. *Arch. Biochem. Biophys.* **1978**, *191*, 444.
(34) Pearce, L. L.; Kurtz, D. M., Jr.; Xia, Y.-M.; Debrunner, P. G. *J. Am. Chem. Soc.*, in press.
(35) We note that rate constants for $H_2O \rightleftharpoons H^+ + OH^-$, $k_f = 2.3 \times 10^{-5}$ s^{-1} and $k_b = 1.3 \times 10^{11}$ M^{-1} s^{-1}, and estimates for those of $[Fe(H_2O)_6]^{3+} \rightleftharpoons [Fe(H_2O)_5OH]^{2+} + H^+$, $k_f = 10^7$–10^8 s^{-1} and $k_b = 10^{10}$–10^{11} M^{-1} s^{-1}, indicate a range of possible dissociation (k_f) values. See e.g.: *Fast Reactions in Solution*; Blackwell Scientific: Oxford, England, 1965; p 75.
(36) Harrington, P. C.; Muhoberac, B. B.; Wharton, D. C.; Wilkins, R. G. *Biochemistry* **1981**, *21*, 6134.
(37) Williams, N. H.; Yandell, J. K. *Aust. J. Chem.* **1983**, *36*, 2377.
(38) Wherland, S.; Gray, H. B. In *Biological Aspects of Inorganic Chemistry*; Addison, A. W., Cullen, W. R., Dolphin, D., James, B. R., Eds.; Wiley: New York, 1977; p 289.
(39) E.g.: Geselowitz, D.; Taube, H. *Adv. Inorg. Bioinorg. Chem.* **1982**, *1*, 391.
(40) Bradič, Z.; Tsukahara, K.; Wilkins, P. C.; Wilkins, R. G. In *Frontiers in Bioinorganic Chemistry*; Xavier, A. V., Ed.; Verlag Chemie: Weinheim, FRG, 1986; p 360.
(41) Armstrong, G. D.; Sykes, A. G. *Inorg. Chem.* **1986**, *25*, 3514.

is influential at pH 6.3. One possibility for *T.z.* is the locality defined by His58, with (nearby) conserved polypeptide lysines at positions 74 and 75, which are close to the active site (His73 is a ligand). In the case of *P.g.* there is no His at position 58 and the negatively charged Glu at 76 presumably makes this region less influential. The fact that $[Co(dipic)_2]^-$ does not give saturation kinetics rules out the possibility that there is a rate-controlling process involving the protein (P → P*) prior to oxidation. We see no reason to favor the so-called "dead-end" mechanism.[44]

Finally it is of interest to make further comparisons of the reactivities of *T.z.* and *P.g.* hemerythrins. Whereas rate constants for the reduction of octameric metHr by $[Co(sep)]^{2+}$ and $[Co(9\text{-}aneN_3)_2]^{2+}$ are very similar, those for $[Fe(CN)_6]^{3-}$ oxidation of deoxy (*P.g.*) are significantly slower ($\sim 10^3$ times at pH 8.2 and 500 times at pH 6.3). It is possible that the faster reaction of $[Fe(CN)_6]^{3-}$ with deoxy (*T.z.*) ensures that isomerization and not redox is rate-determining in the second stage (Scheme II).

Acknowledgment. We thank the Science and Engineering Research Council (U.K.) for support (to G.D.A.) and wish to acknowledge helpful discussions and correspondence from Professors D. M. Kurtz, Jr., and R. G. Wilkins.

Registry No. $[Co(dipic)_2]^-$, 71605-21-5; $[Ru(NH_3)_5(CH_3CN)]^{3+}$, 44819-54-7; $[Fe(CN)_6]^{3-}$, 13408-62-3; $[Co(sep)]^{3+}$, 72496-77-6.

(42) See e.g. sequences in: Sykes, A. G. *Adv. Inorg. Bioinorg. Mech.* **1982**, *1*, 156.
(43) Harrington, P. C.; Wilkins, R. G. *J. Inorg. Biochem.* **1983**, *19*, 334.
(44) See e.g. discussion in: Armstrong, F. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1978**, *100*, 7710.

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Metalloporphyrin Effects on Properties of DNA Polymers

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Interactions of metallo derivatives of *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin [TMpyP(4)] and *meso*-tetrakis(2-*N*-methylpyridiniumyl)porphyrin [TMpyP(2)] with several native and synthetic DNAs were studied by a variety of physical techniques: viscosity, flow dichroism (FD), and NMR (^{31}P and ^1H). The porphyrins were divided into two groups, group I and group III, based on the criteria suggested by Banville et al. Group I porphyrins include NiTMpyP(4) and PdTMpyP(4). Large negative reduced dichroisms (^{red}D) observed in FD studies indicated that the group I porphyrins were bound perpendicular to the axis of calf thymus DNA. ^{31}P NMR spectra of salmon sperm DNA with the group I porphyrins revealed a small broad downfield peak centered at ca. −1 ppm. No significant shifts were noted for the imino proton signals (ca. 12–14 ppm) of salmon sperm DNA on addition of the group I porphyrins. Comparison of the effects of the group I porphyrins on DNAs with different GC content revealed larger changes in solution viscosity with increased GC content. Viscosity changes of AT-rich DNAs were dramatically lower, and precipitation of the DNA–porphyrin adducts was often observed. For poly[d(A-C)(G-T)], a large increase was found in the solution viscosity upon treatment with NiTMpyP(4), raising the possibility of mixed GC/AT intercalation sites for NiTMpyP(4). In addition, NiTMpyP(4) increased the viscosity of calf thymus DNA (CT DNA) to a greater extent than PdTMpyP(4) or TMpyP(4). Contrary to previously reported studies, our viscosity data suggest that NiTMpyP(4) is less selective than TMpyP(4) in intercalative type binding to GC base pairs of DNA; i.e., NiTMpyP(4) also binds to mixed AT/GC sites. However, our viscosity results with PdTMpyP(4) are similar to those reported with TMpyP(4), indicating similar binding properties. Group III porphyrins include NiTMpyP(2), CoTMpyP(4), FeTMpyP(4), ZnTMpyP(4), and SnTMpyP(4). No viscometric increase, low ^{red}D values, and the absence of signals shifted downfield or upfield in ^{31}P and ^1H NMR spectra, respectively, indicate outside, randomly oriented binding. SnTMpyP(4) was shown to nick CCS DNA. This nicking requires the presence of light and may be a radical process dependent on oxidation–reduction of the porphyrin ring system. In any case, it is likely that previously reported changes in the viscosity of CCS DNA with group III porphyrins could be the result of nicking and not intercalation. Thus, all the results we have with group III porphyrins are suggestive of outside binding. The binding of group I porphyrins to DNA is complex, and decreases in signal area in both ^{31}P and ^1H NMR spectra are attributed to outside self-stacking, which leads to aggregation of the polymer at the high concentrations needed for NMR studies. Such signal loss is not usually observed for group III porphyrins, which cannot self-stack. However, in a few cases, some signal loss was evident, and this result was attributed to paramagnetic species. In general, the binding properties of metalloporphyrins appear to reflect those of nonmetalloporphyrins; i.e., the results with group I species are consistent with intercalation whereas those with group III species are most consistent with electrostatic interactions. However, it is not possible to extrapolate the results found with oligonucleotides to those found with these polymers.

Introduction

Binding between DNA and DNA constituents with tetracationic porphyrins and metalloporphyrins has been the subject of numerous recent investigations. Many different techniques (^1H NMR,[1-4] ^{31}P NMR,[1,3,4] circular dichroism,[5-10] viscosity,[1,3,5,6,8,11] fluorescence,[12] flow dichroism,[1] electrophoresis,[11,12,14] electron spin resonance,[15] melting studies,[6] UV–vis[2,6-9,13,16] and resonance Raman[17] spectroscopy, and kinetics[13]) have been applied to a multitude of different polymers and, to a lesser extent, to monomers[2,17] and oligomeric nucleic acid species.[4] However, physical methods used in studies with metalloporphyrins have largely been limited to electronic spectroscopy.

We have recently reported two detailed studies of *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin (TMpyP(4)) (Figure 1). These studies extended the range of nucleic acid polymers that have been studied and included the most extensive NMR

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(1) Banville, D. L.; Marzilli, L. G.; Strickland, J. A.; Wilson, W. D. *Biopolymers* **1986**, *25*, 1837.
(2) Pasternack, R. F.; Gibbs, E. S.; Gaudemer, A.; Antebi, A.; Bassner, S.; DePoy, L.; Turner, D. H.; Williams, A.; Laplace, F.; Lamsurd, M. H.; Merienne, C.; Perree-Favet, M. *J. Am. Chem. Soc.* **1985**, *107*, 8179.
(3) Banville, D. L.; Marzilli, L. G.; Wilson, W. D. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 148.
(4) Marzilli, L. G.; Banville, D. L.; Zon, G.; Wilson, W. D. *J. Am. Chem. Soc.* **1986**, *108*, 4188.
(5) Fiel, R. J.; Carvlin, M. J.; Byrnes, R. W.; Mark, E. H. *Mol. Basis Cancer, Proc. Conf., 1984, Part B.* **1985**, 215.
(6) Fiel, R. J.; Howard, J. C.; Mark, E. H.; Datta Gupta, N. *Nucleic Acids Res.* **1979**, *6*, 3093.
(7) Carvlin, M. J.; Mark, E.; Fiel, R. J.; Howard, J. C. *Nucleic Acis Res.* **1983**, *11*, 6141.
(8) Carvlin, M. J.; Fiel, R. J. *Nucleic Acids Res.* **1983**, *11*, 6121.
(9) Pasternack, R. F.; Gibbs, E. J.; Villafranca, J. J. *Biochemistry* **1983**, *22*, 2406.

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