by filtration, washed with cold water, and dried over P_4O_{10} in vacuo. The compound was recrystallized from cold dichloromethane–hexane; yield 80%. Anal. Calcd for $Na((C_6H_5)_4As)Ru(C_6H_5C(S)NO)_3 \cdot H_2O$: C, 55.21; H, 3.78; N, 4.29. Found: C, 55.10; H, 3.80; N, 4.37.

Acknowledgment. Financial assistance received from the Department of Science and Technology and the Council of Scientific and Industrial Research, New Delhi, is gratefully acknowledged. Thanks are due to Dr. Alok Mukherjee for the X-ray powder diffraction measurements.

Registry No. $Ru(mePh)_3$, 97751-77-4; $Ru(meAn)_3$, 97751-78-5; $Ru(MeBz)_3$, 97751-79-6; $[Ru(mePh)_3]ClO_4$, 97751-81-0; $[Ru(meAn)_3]ClO_4$, 97751-83-2; $[Ru(meBz)_3]ClO_4$, 97751-85-4; $Na[Ph_4As]_2[Ru(ph)_3]$, 97751-87-6; $Na[Ph_4As][Ru(ph)_3]$, 97751-89-8; $Ru(mePh)_3^-$, 97751-90-1; $Ru(meAn)_3^-$, 97751-91-2; $Ru(meBz)_3^-$, 97751-92-3; $Ru(ph)_3^{4-}$, 97751-93-4.

Contribution from the Department of Inorganic Chemistry, The University, Newcastle upon Tyne NE1 7RU, England

Active-Site Chemistry of Hemerythrin: Kinetic Studies on the Reduction of the Met Octamer Form from *Themiste zostericola* with $[Co(sep)]^{2+}$, $[Co(sarCl_2)]^{2+}$, $[Co(9\text{-}aneN_3)_2]^{2+}$, and $[Cr(bpy)_3]^{2+}$

GRAEME D. ARMSTRONG, T. RAMASAMI, and A. GEOFFREY SYKES*

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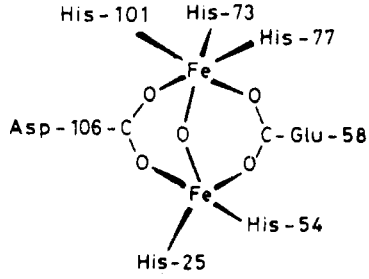
The kinetics of reduction of the binuclear Fe(III,III) active site in the octameric metHr from *Themiste zostericola* through to the Fe(II,II) deoxy form by 1-equiv reductants $[Co(sep)]^{2+}$, $[Co(sarCl_2)]^{2+}$, $[Co(9\text{-}aneN_3)_2]^{2+}$, and $[Cr(bpy)_3]^{2+}$ have been studied at 25 °C, I = 0.15 M (Na_2SO_4). Three stages are observed, which require ~12 h to proceed to completion. The stopped-flow first stage conforms to a rate law first order in both reactants, with rate constants ($k_1/M^{-1}\,s^{-1}$) for $[Co(sep)]^{2+}$ (255), $[Co(sarCl_2)]^{2+}$ (114), $[Co(9\text{-}aneN_3)_2]^{2+}$ (12.8), and $[Cr(bpy)_3]^{2+}$ (2.5×10^5). Uniphasic kinetics are observed over the pH range 6.3–9.0, indicating that the reaction is insensitive as to whether hydroxomet (formed in a slow process $pK_a \sim 8$) is present or not. The pH dependences of k_1 indicate an additional (fast) pK_a of 7.6. Rate constants k_{2obsd} for the second stage ($2.0 \times 10^{-3}\,s^{-1}$ at pH 6.3) are independent of the concentration and identity of the reductant, and give a pK_a close to that for k_1. The third stage k_{3obsd} ($1.2 \times 10^{-4}\,s^{-1}$) is independent of reductant and pH. With dithionite as reductant, the same k_{2obsd} and k_{3obsd} values are obtained. It has been demonstrated that 8, 4, and (by inference) 4 equiv of reductant, respectively, are consumed in each of the three stages required for complete reduction of the octamer. It is concluded that the Fe(II,III) units present in the semi-met form at the end of the first stage have a structure different from the four remaining in the quarter-met at the end of the second stage. Strong reductants $[Cr(edta)]^{2-}$ and dithionite were also used to generate UV–vis spectra of intermediate states.

Introduction

Hemerythrin is one of three naturally occurring O_2 carriers.[1-5] It is found in four different invertebrate phyla, the sipunculids, brachiopods, polychaetes, and priapulids, of which the former are the major source and the most extensively studied. Hemerythrin for this work was obtained from the spinuculid marine worm *Themiste zostericola*. The octamer (mol wt 108 000) obtained from the erythrocytes consists of eight identical subunits each of which has a binuclear Fe non-heme active site. Little or no cooperativity is observed for the extracted octamer, and the reason for its existence in this state is not at present understood. A monomer has also been isolated from the retractor muscle and will be the subject of further studies.

Recent X-ray studies on crystals of Fe(III,III) methemerythrin from *Themiste dyscritum* (pH ≤6.5) have indicated a structure in which one of the Fe(III) atoms is octahedral and the other is trigonal bipyramidal:[6]



Coordination of azide is known to occur at the sixth (vacant) position, and O_2 is believed to bind to the deoxy form, Fe(II,II), at this same site giving a product that can be described as peroxo Fe(III,III). It has recently been demonstrated that OH^- coordinates to the 5-coordinate Fe of the met form in a slow acid–base equilibrium,[7,8] the pK_a of which is 7.8 for *Phascolopsis gouldii* and 8.4 for *Themiste dyscritum*. Similar reactivity is observed for the met form from *Themiste zostericola*. EXAFS,[9] Mössbauer,[10] and resonance Raman[11] studies support a μ-oxo-bridged structure for both met and oxy forms, and structural features include Fe–O(oxo) (*ca.* 1.75 Å) and Fe–Fe (ca. 3.3 Å) bonds and an Fe–O–Fe angle of 165°. In the deoxy form the two Fe(II)'s are not coupled antiferromagnetically,[10,12] and EXAFS studies suggest that there is no μ-oxo bridge.[9] This question is also addressed by Reem and Solomon.[13]

In order to better understand the chemistry of the hemerythrin active site, which is now believed to function also in purple acid phosphatase[14] and ribonuclease,[15] we have commenced a program of study in which redox interconversions are investigated in more detail. Wilkins and colleagues have studied previously the reduction of metHr with dithionite[16] and have successfully characterized by EPR a semi-met intermediate.[17] Other studies by

(1) Wood, E. J. *Essays Biochem.* **1980**, *16*, 1.
(2) Sykes, A. G. *Adv. Inorg. Bioinorg. Mech.* **1982**, *1*, 154–167.
(3) Collman, J. P.; Halbert, T. R.; Suslick, K. S. In "Metal Ion Activation of Dioxygen"; Spiro, T. G., Ed.; Wiley: New York; 1980; p 1.
(4) Klotz, I. M.; Kurtz, D. M. *Acc. Chem. Res.* **1984**, *17*, 16.
(5) Wilkins, R. G.; Harrington, P. C. *Adv. Inorg. Biochem.* **1983**, *5*, 51–85.
(6) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H. *J. Am. Chem. Soc.* **1984**, *106*, 618.
(7) Bradič, Z.; Wilkins, R. G. *Biochemistry* **1983**, *22*, 5396.
(8) McCallum, J. D.; Shiemke, A. K.; Sanders-Loehr, J. *Biochemistry* **1984**, *23*, 2819.
(9) (a) 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. (b) Elam, W. T.; Stern, E. A.; McCallum, J. D.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1982**, *104*, 6369, **1983**, *105*, 1919.
(10) Garbett, K.; Johnson, C. L.; Klotz, I. M.; Okamura, M. Y.; Williams, R. J. P. *Arch. Biochem. Biophys.* **1971**, *142*, 574.
(11) Freier, S. M.; Duff, L. L.; Shriver, D. F.; Klotz, I. M. *Arch. Biochem. Biophys.* **1980**, *205*, 449.
(12) Klotz, I. M.; Klippenstein, G. L.; Hendrickson, W. A. *Science (Washington, DC)* **1976**, *198*, 335.
(13) Reem, R. C.; Solomon, E. I. *J. Am. Chem. Soc.* **1984**, *106*, 8323.
(14) Davis, J. C.; Averill, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 4623.
(15) Sjöberg, B.-M.; Loehr, T. M.; Sanders-Loehr, J. *Biochemistry* **1982**, *21*, 96.
(16) Harrington, P. C.; DeWaal, D. J. A.; Wilkins, R. G. *Arch. Biochem. Biophys.* **1978**, 191, 441.

0020-1669/85/1324-3230$01.50/0

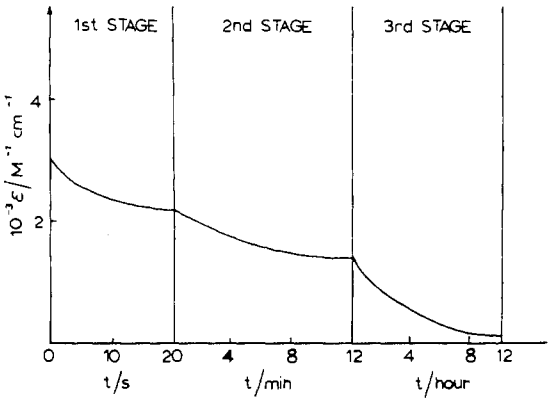


Figure 1. Absorbance changes at 25 °C, λ = 400 nm showing the three stages for the reduction of metHr (1×10^{-4} M) with $[Co(sep)]^{2+}$ (1.4×10^{-3} M), at pH 6.3 (Mes), I = 0.15 M (Na_2SO_4).

Irwin et al.[18] have confirmed essential characteristics of this state.

Experimental Section

Preparation of Protein. The octamer of oxyHr was isolated from the coelomic fluid of *Themiste zostericola* (Pacific Bio-Marine Laboratory, Venice, CA) by using a modified version of the procedure described by Klippenstein et al.[19] (dialysis against NaN_3 was omitted). 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. To avoid complications arising from the slow adjustment of the protein to new pHs, it was necessary to leave the protein for at least 15 h at 4 °C to equilibrate. Throughout this paper concentrations of protein are expressed as monomer[20] and were established by using absorption measurements at 333 nm (ϵ = 6500 M^{-1} cm^{-1} per monomer), which is an isosbestic point for the acid–base forms of metHr.

Rigorous air-free conditions were employed in all studies. Protein solutions were deoxygenated by dialysis against deaerated buffer. Nitrogen gas (BOC, White Spot grade), further purified by bubbling through solutions of Cr^{2+}, was used in all experiments. Procedures adopted avoided bubbling N_2 through hemerythrin solutions, which resulted in protein denaturation.

Preparation of Complexes. These were obtained and purified to known spectral peak positions λ/nm (ϵ/M^{-1} cm^{-1}) by procedures already described: $[Co(sep)]Cl_3 \cdot H_2O$, where sep denotes the sepulchrate cage ligand 1,3,6,8,10,13,16,19-octaazabicyclo[6.6.6]eicosane, 472 (109), 340 (116);[21] $[Co(sarCl_2)]Cl_3$, where $sarCl_2$ is the cage ligand 1,8-dichloro-3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosane, 474 (143), 345 (121);[22] $[Co(9\text{-}aneN_3)_2]Cl_3 \cdot 3H_2O$, where 9-$aneN_3$ is 1,4,7-triazacyclononane, 458 (100), 333 (89);[23] chromium(II) chloride, $CrCl_2 \cdot 6H_2O$, 715 (4.8);[24] tris(1,10-phenanthroline)iron(III) perchlorate, $[Fe(phen)_3](ClO_4)_3 \cdot H_2O$,[25] which was determined by reduction with Zn/Hg to the $[Fe(phen)_3]^{2+}$ peak at 510 (10 900). Samples of $[Co(sarCl_2)]Cl_3$ and $[Co(9\text{-}aneN_3)_2]Cl_3 \cdot 3H_2O$ were generously provided by Professors A. M. Sargeson and K. Wieghardt. The three Co(III) complexes were reduced to Co(II) at a Hg-pool electrode by controlled potentiometry with a Chemical Electronics Ltd. (Washington, Tyne and Wear) potentiostat, Model DD505U. No variation in rate constants was observed when sulfate salts of $[Co(sep)]^{2+}$ and $[Co(9\text{-}aneN_3)_2]^{2+}$ were used instead of the chloride salts.

Other complexes, tris(1,10-phenanthroline)chromium(II), $[Cr(phen)_3]^{2+}$, and tris(2,2'-bipyridine)chromium(II), $[Cr(bpy)_3]^{2+}$, $E°$ = −0.28 and −0.26 V, respectively,[26] were generated in solution by addition of an excess (≥10-fold) of ligand (Aldrich Chemical Co.) to a solution of Cr^{2+}. Reductant concentrations were determined by titration against $[Fe(phen)_3]^{3+}$, when the $[Fe(phen)_3]^{2+}$ product was determined spectrophotometrically. Solutions of $[Cr(edta)]^{2-}$ were obtained by addition of Cr^{2+} to an 8-fold excess of disodium ethylenediaminetetraacetate (Aldrich) in Mes and Tris buffers as shown below. Sodium dithionite (BDH, AnalaR) was determined by titration against solutions of $K_3[Fe(CN)_6]$, peak at 420 nm, ϵ = 1010 M^{-1} cm^{-1}.

Buffers. A 0.050 M solution of tris(hydroxymethyl)aminoethane (Tris) (Sigma Chemicals) adjusted with 1.0 M H_2SO_4 (BDH, AnalaR) was used as buffer over the range pH 7.0–9.0. The buffer 2-morpholinoethanesulfonic acid (Mes) (Sigma Chemicals) in a 0.050 M solution, to which 1.0 M NaOH (BDH, AnalaR) was added, was used over the range pH 5.0–7.0. A mixed buffer of Tris and maleic acid (BDH, AnalaR) both at 0.050 M and adjusted to the required pH by

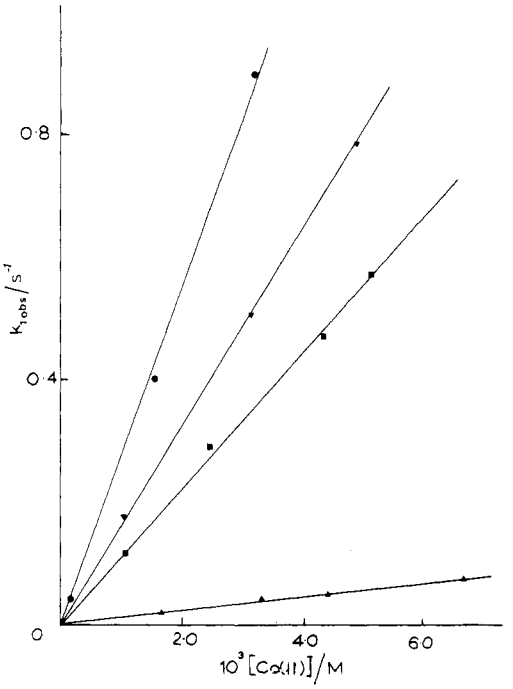


Figure 2. Linear dependence of k_{1obsd} (25 °C) for the reduction of metHr with $[Co(sep)]^{2+}$ (●), $[Co(sarCl_2)]^{2+}$ (■), and $[Co(9\text{-}aneN_3)]^{2+}$ (▲) on reduction concentration at pH 6.3 (Mes), I = 0.15 M (Na_2SO_4). Rate constants for the reduction with $[Co(sep)]^{2+}$ at pH 8.2 (Tris) are also included (▼).

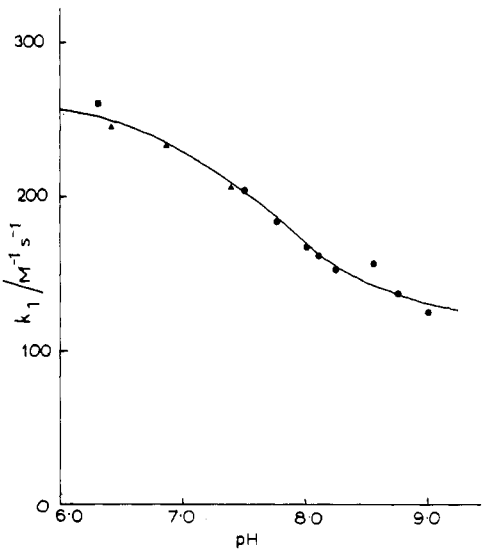


Figure 3. Variation of k_1(25 °C) for the first stage of the $[Co(sep)]^{2+}$ reduction of metHr with pH, I = 0.15 M (Na_2SO_4). Buffers used: Mes (■), Tris/maleate (▲), and Tris (●).

(17) Muhoberac, B. B.; Wharton, D. C.; Babcock, L. M.; Harrington, P. C.; Wilkins, R. G. *Biochem. Biophys. Acta* **1980**, *626*, 337.
(18) Irwin, M. J.; Duff, L. L.; Shriver, D. F.; Klotz, I. M. *Arch. Biochem. Biophys.* **1983**, *224*, 473.
(19) Klippenstein, G. L.; Van Riper, D. A.; Oosterom, E. A. *J. Biol. Chem.* **1972**, *247*, 5959.
(20) Garbett, K.; Darnall, D. W., Klotz, I. M.; Williams, R. J. P. *Arch. Biochem. Biophys.* **1469**, *135*, 419.
(21) 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.
(22) Geue, R. J.; Hambley, T. W.; Harrowfield, J. M.; Sargeson, A. M.; Snow, M. R. *J. Am. Chem. Soc.* **1984**, *106*, 5478.
(23) Wieghardt, K.; Schmidt, W.; Herrmann, W.; Kuppers, H.-J. *Inorg. Chem.* **1983**, *21*, 2953.
(24) Holah, D. G.; Fackler, J. P., Jr. *Inorg. Synth.* **1969**, *10*, 26.
(25) Shildt, A. A.; Taylor, R. C. *J. Inorg. Nucl. Chem.* **1959**, *9*, 211.
(26) Brunschwig, B.; Sutin, N. *J. Am. Chem. Soc.* **1978**, *100*, 7568.
(27) See, e.g.: Henderson, R. A.; Sykes, A. G. *Inorg. Chem.* **1980**, *19*, 3103.

Table I. Effect of pH on Rate Constants k_1 (25 °C) for the First Stage of Reduction of *T. zostericola* Methemerythrin (ca. 5×10^{-5} M), I = 0.15 M (Na_2SO_4)

reductant	pH	k_1, M^{-1} s^{-1}
$[Co(sep)]^{2+}$	6.30 (Mes)	260
	6.39 (Tris/Mal)	242
	6.85 (Tris/Mal)	233
	7.35 (Tris/Mal)	208
	7.50 (Tris/Mal)	205
	7.75 (Tris)	185
	8.00 (Tris)	164
	8.05 (Tris)	164
	8.20 (Tris)	153
	8.55 (Tris)	159
	8.75 (Tris)	138
	9.00 (Tris)	122
$[Co(9\text{-}aneN_3)_2]^{2+}$	6.30 (Mes)	12.2
	6.90 (Tris/Mal)	11.6
	7.41 (Tris)	9.5
	8.26 (Tris)	8.2
	8.57 (Tris)	7.4
$[Co(sarCl_2)]^{2+}$	6.30 (Mes)	113
	6.85 (Tris/Mal)	107
	7.08 (Tris)	99
	8.09 (Tris)	74
	8.64 (Tris)	60

addition of 1.0 M NaOH, was used to complete the pH range 6.0–7.5. Examination of pH effects such as illustrated in Figure 3 indicate no effect of buffer or reductant. All buffer solutions were adjusted to an ionic strength of 0.150 ± 0.001 M with anhydrous Na_2SO_4 (BDH, AnalaR). The pH of solutions was determined by using a Radiometer (PHM 62) pH meter with a Russell (CWR/322) glass electrode.

Kinetics. Fast stages, $k_{obsd} > 10^{-2}$ s^{-1}, were monitored by using a Dionex D-110 stopped-flow spectrophotometer, and absorbance traces were stored on a Tektronix 5150 storage oscilloscope. Slow reactions were monitored by conventional spectrophotometry using either a Perkin-Elmer 554 or Lambda 5 UV–vis spectrophotometer. Inorganic reactants were in >10-fold excess of the protein. Reactions were monitored at wavelengths in the range 320–400 nm, depending on the reductant used. The absorbance change was largely (>97%) due to the protein component. A standard consecutive reaction treatment was used.[28] All reactions were studied at 25.0 ± 0.1 °C, with ionic strength, I, adjusted to 0.150 M (Na_2SO_4). Under the conditions employed, the sulfate was present as SO_4^{2-}. Other anions that are normally used to adjust ionic strength, such as Cl^- and ClO_4^-, are reported to associate with Hr.[7,8,29] No anomalous effects of SO_4^{2-} have yet been reported in studies from other groups.

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

Results

Over the pH range 6.3–9.0 investigated, the reduction was found to occur in three stages (Figure 1). The first required the stopped-flow method, and the second and third required conventional spectrophotometry.

Fast Stage. Stopped-flow traces gave satisfactory final absorbance (A_∞) values for the higher reductant concentrations. First-order plots, ln ($A_t - A_\infty$) against time, were linear to >90% reaction and gave rate constants k_{1obsd}. The Guggenheim method[30] was used for runs at lower reductant concentrations when some drift in A_∞ values (due to incidence of the second stage), was observed. Linear dependences on the concentration of reductants were observed (Figure 2), consistent with (1). Due to the extreme

$$k_{1obsd} = k_1[\text{reductant}] \quad (1)$$

oxygen sensitivity of $[Cr(bpy)_3]^{2+}$ (0.9×10^{-5} M) first-order rate constants (k_{1obsd}) 25.0, 10.7, and 20.7 s^{-1} were determined with 10^4[metHr] at 1.25, 0.54, 0.91 M respectively in excess (pH 6.3). Rate constants k_1, which vary with the identity of the reductant,

(28) Frost, A. A.; Pearson, R. G. "Kinetics and Mechanism", 2nd ed.; Wiley: New York, 1961; p 166.
(29) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H. *J. Mol. Biol.* **1978**, *126*, 457.
(30) Reference 28; p 49.

Table II. Summary of Rate Constants k_1(25 °C) for the First Stage of the Reduction of metHr at Either Extreme of pH, Here Designated as k_A (pH <6) and k_B (pH >9), (3)–(4), I = 0.15 M (Na_2SO_4)

reductant	$E°$,[a] V	k_A, M^{-1} s^{-1}	k_B, M^{-1} s^{-1}
$[Co(sep)]^{2+}$	−0.26	255 ± 6	129 ± 6
$[Co(sarCl_2)]^{2+}$	−0.13	114 ± 3	56 ± 5
$[Co(9\text{-}aneN_3)_2]^{2+}$	−0.41	12.8 ± 0.5	7.3 ± 0.4
$[Cr(bpy)_3]^{2+}$	−0.26	(2.5 ± 0.5) × 10^5	

[a] Reduction potentials of III/II couples. References as in text.

Table III. Rate Constants, k_{2obsd}(25 °C), for the Second Stage of the Reduction of *T. zostericola* metHr (7.5×10^{-5} M) with $[Co(sep)]^{2+}$, $[Co(sarCl_2)]^{2+}$, and Dithionite, I = 0.15 M (Na_2SO_4)

reductant	pH	10^3[reductant], M	$10^3 k_{2obsd}$, s^{-1}
$[Co(sep)]^{2+}$	6.30 (Mes)	5.0	2.11
	6.30 (Mes)	1.40	2.05
	6.30 (Mes)	0.75	2.08
$[Co(sarCl_2)]^{2+}$	6.30 (Mes)	1.40	2.20
	7.00 (Tris)	5.0	1.40
	7.00 (Tris)	1.40	1.33
dithionite	6.30 (Mes)	0.50	1.97
	8.20 (Tris)	0.50	3.90
	8.20 (Tris)	2.50	3.90

Table IV. Effect of pH on Rate Constants, k_{2obsd}(25 °C), for the Second Stage of the Reduction of *T. zostericola* metHr, (1.2–0.75) × 10^{-5} M, with $[Co(sep)]^{2+}$ and $[Co(sarCl_2)]^{2+}$, I = 0.15 M (Na_2SO_4)

reductant	pH	$10^3 k_{2obsd}$, s^{-1}	pH	$10^3 k_{2obsd}$, s^{-1}
$[Co(sep)]^{2+}$	9.00 (Tris)	3.9	7.28 (Tris)	1.77
	8.64 (Tris)	4.2	6.96 (Tris/Mal)	1.32
	8.09 (Tris)	3.5	6.50 (Mes)	1.65
	7.67 (Tris)	2.70	6.40 (Mes)	1.80
	7.60 (Tris)	2.30	6.30 (Mes)	2.05
	7.60 (Tris)	2.15	6.10 (Mes)	2.63
$[Co(sarCl_2)]^{2+}$	8.90 (Tris)	3.9	7.65 (Tris)	2.89
	8.40 (Tris)	3.8	7.00 (Tris)	1.33
	8.00 (Tris)	2.96	6.30 (Mes)	2.20

Table V. Rate Constants, k_{3obsd}(25 °C), for the Third Stage of the Reduction of *T. zostericola* metHr (9.5×10^{-5} M) with $[Co(sep)]^{2+}$, $[Co(sarCl_2)]^{2+}$, and Dithionite, I = 0.15 M (Na_2SO_4)

reductant	pH	10^3[reductant], M	$10^4 k_{3obsd}$, s^{-1}
$[Co(sep)]^{2+}$	6.30 (Mes)	3.4	1.2
	8.20 (Tris)	3.4	1.3
	8.20 (Tris)	0.8	1.2
	8.80 (Tris)	3.4	1.3
$[Co(sarCl_2)]^{2+}$	6.30 (Mes)	2.2	1.0
dithionite	6.30 (Mes)	0.5	1.0
	8.20 (Tris)	0.5	1.1
	8.20 (Tris)	2.5	1.0

also vary with pH (Table I), as illustrated in Figure 3 for $[Co(sep)]^{2+}$. The reaction sequence (2)–(4), which gives the de-

$$HrH^+ \rightleftharpoons Hr + H^+ \quad (2)$$

$$HrH^+ + C \xrightarrow{k_A} \text{products} \quad (3)$$

$$Hr + C \xrightarrow{k_B} \text{products} \quad (4)$$

pendence shown in (5), provides a good fit of the data. Values

$$k_{1obsd} = \frac{k_A[H^+] + k_B K_a}{[H^+] + K_a} \quad (5)$$

of k_1 at the two extremes of this pH range (k_A and k_B) are listed in Table II. The combined pK_a for three reductants is 7.6 ± 0.2.

Second and Third Stages. Rate constants k_{2obsd} (Tables III and IV) and k_{3obsd} (Table V) were obtained by applying a consecutive treatment to the second and third stages. These were found to

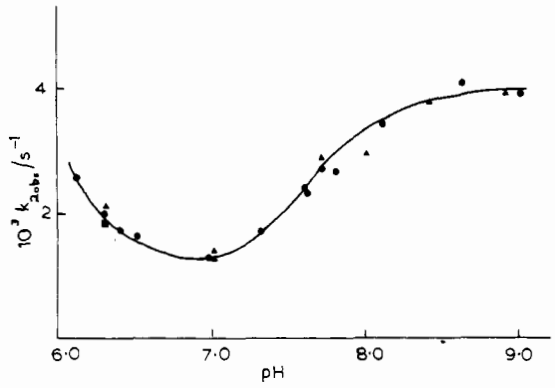


Figure 4. Variation of k_{2obsd} (25 °C) for the second stage of the [Co(sep)]$^{2+}$ (●), [Co(sarCl$_2$)]$^{2+}$ (▲), and [Cr(phen)$_3$]$^{2+}$ (■) reduction of metHr with pH, I = 0.15 M (Na_2SO_4).

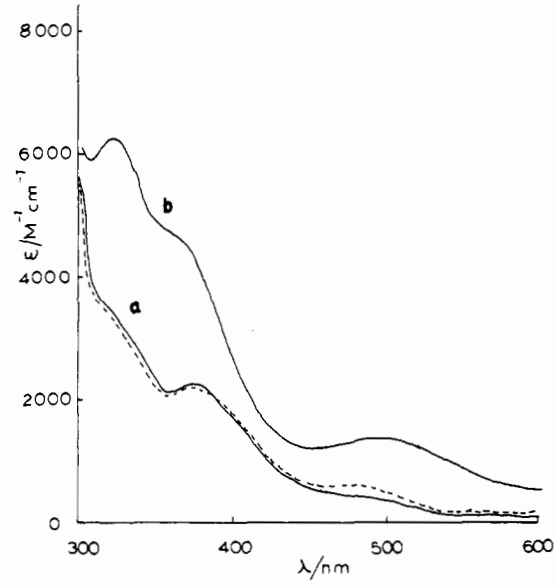


Figure 5. Spectrum of the product at the end of the second stage (quarter-met form) (a), and spectrum after reaction of the product with O_2 to give 4 equiv of oxyHr (b). No reaction with azide is observed (broken line).

be independent of the concentration and identity of the reductants. Dithionite, which has been used previously as reductant,[16] also conformed to these studies. A complex dependence on pH is apparent for the second stage (Figure 4). When an equation of the same format as (5) is applied to the higher and lower ranges of pH, pK_{2a}'s of 7.6 (i.e. about the same as for the first stage) and less than 6.0 were estimated. Variations in pH within the range 6.3–8.8 had no effect on rate constants k_{3obsd} (Table V).

Stoichiometry of Different Stages. Consumption of reductant for each stage of reaction was determined from spectrophotometric measurements with 4 × 10^{-5} M met protein, by using as reductant the complex [Cr(phen)$_3$]$^{2+}$ (2 × 10^{-4}M), which has a prominent absorbance peak at 850 nm with $\Delta\epsilon$ for the one-electron oxidation of 3650 M^{-1} cm^{-1}.[26] Results from four determinations indicated 8 equiv of reductant are consumed in the first stage (8.1 ± 0.2), followed by 4 in the second (4.0 ± 0.1). The length of time required for completion of the third stage made accurate quantitative measurements difficult with such an air-sensitive reductant.

It was found that the semi-met product at the end of the first stage did not bind O_2, whereas the product of the second stage binds 4 mol of O_2 (Figure 5). The latter was determined by making use of the prominent oxyHr peak at 500 nm (ϵ = 2200 M^{-1} cm^{-1} per monomer).[19] Complete reduction to deoxyHr, which has negligible absorbance at λ > 400 nm, was observed at the end of the third stage. Confirmation that there was complete reduction was obtained by reacting the product with O_2 and determining the oxyHr formed.

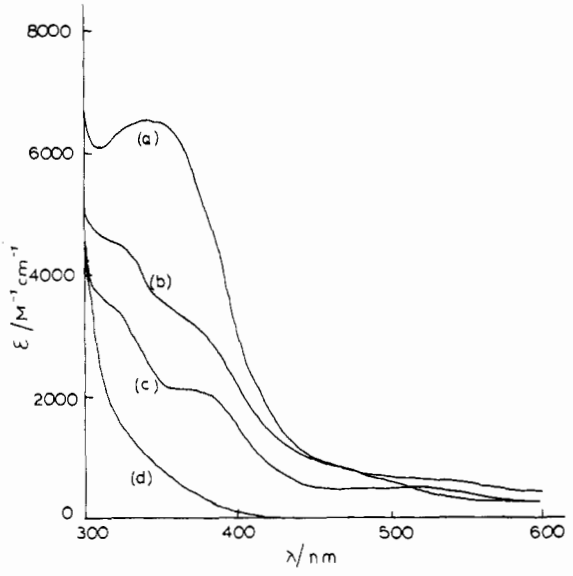


Figure 6. Spectra of (a) metHr, (b) semi-metHr (product at end of first stage), (c) quarter-metHr (product at end of second stage), and (d) deoxyHr (end of third stage) at pH 6.3 (Mes), I = 0.15 M (Na_2SO_4).

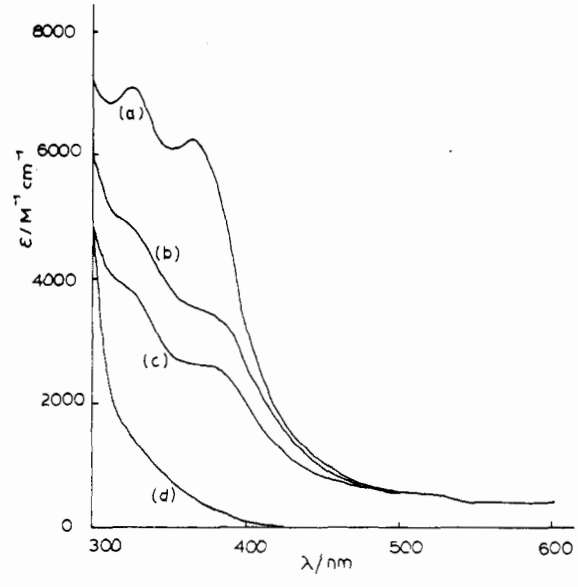


Figure 7. Spectra of (a) metHr, (b) semi-metHr (product at end of first stage), (c) quarter-metHr (product at end of second stage), and (d) deoxyHr (end of third stage) at pH 8.2 (Tris), I = 0.15 M (Na_2SO_4).

The product at the end of stage 1 reacts with azide, giving a large absorbance change at 470 nm.[16] No absorbance changes were observed between 300 and 600 nm (Figure 5) for reaction of the product at the end of stage 2 with azide (3 × 10^{-3} M) at pH 6.3 and 8.2.

Spectra. UV–vis spectra were recorded for the products at the end of the first and second stages of reduction of metHr (1 × 10^{-4} M) at pH 6.3 (Figure 6) and pH 8.2 (Figure 7) with [Cr(edta)]$^{2-}$, which is a sufficiently strong reductant to bring about an essentially instant reduction. The spectrum of the quarter-met second-stage product was generated by the addition of >12 equiv of [Cr(edta)]$^{2-}$ per octamer, thus confirming the stoichiometric measurements made with [Cr(phen)$_3$]$^{2+}$. Identical spectra corresponding to completion of stages 1 and 2 were generated by addition of dithionite. Spectra[31] in an earlier communication for pH 6.3 are to be replaced by those in Figure 6, which represent a further refinement of data.

(31) Armstrong, G. D.; Ramasami, T.; Sykes, A. G. *J. Chem. Soc., Chem. Commun.* **1984**, 1017.

Discussion

The reduction of octameric metHr occurs in three stages (Figure 1). Reduction to a semi-met $Fe(II,III)_8$ form, previously described by Wilkins and colleagues,[17] is observed in the first stage in which 8 equiv of reductant are consumed. Comparison of second-order rate constants k_1 for the reduction with $[Cr(bpy)_3]^{2+}$ and $[Co(sep)]^{2+}$ are consistent (within a factor of 10) with $E°$ values (Table II) self-exchange rate constants of 2×10^9 M^{-1} s^{-1} and 5.1 M^{-1} s^{-1} for the $[Cr(bpy)_3]^{2+/3+}$ and $[Co(sep)]^{2+/3+}$ couples, respectively.[26,21] However, from known reactivities $[Co(sep)]^{2+}$ might have been expected to react more slowly than $[Co(9\text{-}aneN_3)_2]^{2+}$, which is not the case. The active site of each subunit is known to be buried deep in the protein interior,[32] and it is possible that the reductant may choose to partly enter the channel designed for O_2 to gain access to the active site. If this is the case, then the size of the complex interactions with residues on the protein could influence the rate constants k_1 for electron transfer.

Further reduction of the semi-met $Fe(II,III)_8$ form is controlled by isomerization, which, it has been suggested, involves intramolecular electron transfer over ~30 Å yielding Fe(III,III) and Fe(II,II) units.[33] Rapid electron transfer to Fe(III,III) by an external reductant may then occur as in the first stage. This mechanism does not explain the fate of the last Fe(II,III) unit, however, nor is any intramolecular process possible when myoHr is the reactant. The high absorbance at the end of the second stage is unlikely to be accounted for by the last remaining Fe(II,III) and from the stoichiometry measurements reported in this paper is due to four Fe(II,III)'s in a quarter-met form. Reductions with $[Co(sep)]^{2+}$, $[Co(sarCl_2)]^{2+}$, and dithionite have shown that rates for the second and third stages are not dependent on the nature (or concentration) of the reductant, consistent with intramolecular processes.

The existence of a quarter-met form $Fe(II,III)_4Fe(II,II)_4$ cannot be explained in terms of the existing mechanism, unless reduction in the second stage is accompanied by a conformational change, which produces an Fe(II,III) unit different from that generated in the first stage. Whereas Fe(II,III) in the $Fe(II,III)_8$ product reacts with azide, no similar reaction is observed for Fe(II,III) in the quarter-met form. Reaction of the quarter-met form with O_2 gives four oxyHr, in support of the stoichiometry and formula given. Reduction in the third stage differs from that in the second in that there is no dependence on $[H^+]$, consistent with structure differences. A comparison of rate constants for the second stage of reduction of octamer (3.7×10^{-3} s^{-1}) and myoHr (4.0×10^{-3} s^{-1} unpublished work) at pH 8.2 suggests a common intramolecular process involving Fe(II,III) and implies that electron transfer over ~30 Å does not constitute the rate-controlling step. This and other implications of studies on myoHr will be the subject of a further paper.

A slow interconversion of acid and base forms of metHr (requiring 1 h) has been reported.[7] X-ray crystallographic information for the acid form (from *Themiste dyscritum*) has shown that the active site consists of one octahedral and one trigonal-bipyramidal Fe.[6] No H_2O ligand is coordinated to the active site, and the aquamet terminology that has sometimes been used is not therefore appropriate. At pH >9 resonance Raman and difference spectroscopy have demonstrated that a hydroxo ligand is present.[8] It is likely therefore that the 5-coordinate Fe is converted to a 6-coordinate hydroxo form with OH^- occupying the position that N_3^- and, in oxyHr, O_2 can occupy. The simplest way in which this could occur is by a two-stage mechanism, (6)–(7), or alternatively by synchronous loss of a proton at the time of H_2O coordination as in (8). Equation 8 will be adopted

$$Hr + H_2O \rightleftharpoons HrH_2O \quad (6)$$

$$HrH_2O \rightleftharpoons HrOH^- + H^+ \quad (7)$$

$$Hr + H_2O \overset{K_a}{\rightleftharpoons} HrOH^- + H^+ \quad (8)$$

for the present since it represents the simplest possible way in which to summarize the reaction. From spectroscopic measurements, pK_a values of 7.8 and 8.4 have been reported for hemerythrin from *Phascolopsis gouldii*[7] and *Themiste dyscritum*,[8] respectively, and a value of 7.8 has been observed in preliminary studies on *Themiste zostericola*. Since this equilibrium is slow and gives rise to different UV–vis spectra (see upper spectra for metHr at pH 6.3 and 8.2 in Figures 6 and 7, respectively), biphasic kinetics would be expected in the first stage of the (stopped-flow) reduction of metHr at pH's in the range 6.3–9.0. No such behavior is observed (linearity of first-order plots >90%), but instead a rapidly established pK_a of 7.6 is apparent (Figure 3). Experiments with $[Cr(phen)_3]^{2+}$ at pH 6.3 and with $[Cr(edta)]^{2-}$ and dithionite at pH 6.3 and 8.2 have indicated the same stoichiometry for the different stages, with no dependence on pH. A possible inference is that the 6-coordinate and not the (at low pH) 5-coordinate Fe site is reduced in the first stage, in agreement with NMR contact-shift measurements by Maroney et al.[34] This would imply that the coordination number of the second Fe has no influence on the kinetics. Interestingly, a similar pK_a is observed in the second stage of the reduction, although the effect of pH in Figure 4 (stage 2) is in the opposite direction to that in Figure 3 for stage 1. The rapidly established pK_a of 7.6 can be accounted for by acid dissociation of some adjacent influential amino acid. Recent resonance Raman investigations have suggested that there is hydrogen bonding of the peroxo moiety in oxyhemerythrin to the μ-oxo group.[35] It has been noted that Tyr109 is sufficiently close to be involved in some way.[36] In the met form hydrogen bonding between the μ-oxo ligand and Tyr109 is possible, and would provide a satisfactory explanation, since k_A and k_B for the rapidly equilibrating acid and base forms differ only by a factor of 2. Earlier work has suggested equilibria involving two protons with pK_a's ~ 7.8,[37] although this result has more recently been challenged.[7,38]

To summarize the proposal[33] that there is rate-controlling intramolecular long-distance electron transfer within the semi-met octamer form requires reexamination. The stoichiometry and spectrophotometric measurements described have indicated that interconversions are controlled by changes at the active site that are closely linked to pH effects. Conformational changes are believed to be involved, as has been suggested by Wilkins et al.[7,33] The complexity of redox interconversions is reflected in the extensive structural differences that are apparent from EXAFS studies on the met and deoxy forms.[9]

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Registry No. $[Co(sep)]^{2+}$, 82796-47-2; $[Co(sarCl_2)]^{2+}$, 85664-35-3; $[Co(9\text{-}aneN_3)_2]^{2+}$, 91760-59-7; $[Cr(bpy)_3]^{2+}$, 17632-84-7; dithionite, 14844-07-6.

(32) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H.; McCallum, J.; Sanders-Loehr, J. submitted for publication in *Proc. Natl. Acad. Sci. U.S.A.* Kurtz, D. M.; Shriver, D. F.; Klotz, I. M. *Coord. Chem. Rev.* **1977**, *24*, 145.

(33) Harrington, P. C.; Wilkins, R. G. *J. Am. Chem. Soc.* **1981**, 103, 1550.

(34) Maroney, M. J.; Lauffer, R. B.; Que, L. *J. Am. Chem. Soc.* **1984**, *106*, 6445.

(35) Shiemke, A. K.; Loehr, T. M.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1984**, *106*, 4951.

(36) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H. *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem.* **1982**, *B38*, 784; *J. Inorg. Biochem.* **1983**, *19*, 247.

(37) Garbett, K.; Darnall, D. W.; Klotz, I. M. *Arch. Biochem. Biophys.* **1971**, *142*, 471.

(38) Gorman, E. G.; Darnall, D. W. *Biochemistry* **1981**, *20*, 38.