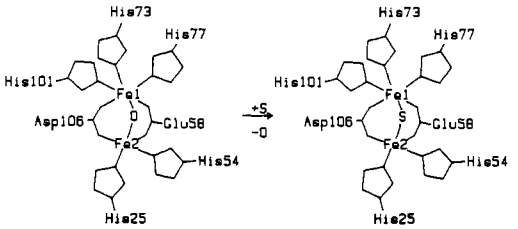
Comparisons of Redox Kinetics of Methemerythrin and μ-Sulfidomethemerythrin. Implications for Interactions with Cytochrome b_5†

Linda L. Pearce and Ronald E. Utecht
Department of Chemistry, Iowa State University, Ames, Iowa 50011
Donald M. Kurtz, Jr.*
Department of Chemistry, University of Georgia, Athens, Georgia 30602
Received April 7, 1987; Revised Manuscript Received July 28, 1987

ABSTRACT: We have examined the effects on redox kinetics of changing the reduction potential of the μ-oxo-bridged binuclear iron center in octameric hemerythrin (Hr) from *Phascolopsis gouldii*. The opportunity to examine such effects is provided by the availability of μ-sulfidomethemerythrin (μ-S^{2-}metHr), whose [Fe(III),Fe(III)]met → [Fe(II),Fe(III)]semi-met reduction potential is ~200 mV higher than that of methemerythrin (metHr). We have used, as redox partners to Hr, a set of metal complexes and the heme proteins deoxymyoglobin (Mb) and cytochrome b_5. The latter protein from *P. gouldii* is a presumed physiological redox partner of Hr. Similar kinetics at pH 8 in the presence or absence of the allosteric effector perchlorate suggest reduction of the iron atom closer to the outer surface of each subunit in the Hr octamer during the met → semi-met transformation. For all reducing agents, the experimentally determined ratio of second-order rate constants for reductions of μ-S^{2-}metHr and metHr, k_{12}(μ-S^{2-}met)/k_{12}(met), is close to the value of 40 predicted by the simple Marcus relation for "outer-sphere" electron transfer. For oxidations of (semi-met)$_R$Hr and μ-S^{2-}semi-metHr, the predicted value of 40 for k_{12}[(semi-met)$_R$]/k_{12}(μ-S^{2-}semi-met) is closely approximated when $Fe(CN)_6^{3-}$ is used as oxidant. The ionic strength dependence of the second-order rate constant suggests electrostatic interactions of opposite charges during reduction of metHr by *P. gouldii* cytochrome b_5. We propose the formation of an electron-transfer complex between cytochrome b_5 and Hr which uses lysine residues on the outer surface of Hr to form salt bridges to carboxylate residues on cytochrome b_5. This hypothetical complex is similar to a proposed electron-transfer complex between Mb and cytochrome b_5. Agreement between observed and calculated values of the ratio k_{12}(cyt b_5–Mb)/k_{12}(cyt b_5–Hr) is consistent with similar types of interactions in these two electron-transfer complexes.

The non-heme iron oxygen-carrying protein hemerythrin (Hr),[1] found in sipunculan worms, presents physiological and biochemical contrasts to the more widespread heme oxygen carriers (Klotz & Kurtz, 1984; Kurtz, 1986). Hr from erythrocytes of sipunculids usually consists of an octamer of essentially identical subunits. Each subunit contains a binuclear iron oxygen binding site. The two iron atoms are linked to the protein by terminal imidazole and bridging carboxylate ligands. In [Fe(III),Fe(III)]metHr, a bridging oxo ion is provided by solvent (Stenkamp et al., 1984; Sieker et al., 1982).



	metHr	μ-S^{2-}metHr
$E^{0\prime}$(III,III→II,III)	110 mV	295 mV

The open coordination position on Fe2 is occupied by hydroperoxide, OOH^-, in oxyHr (Stenkamp et al., 1985) and by any of a number of small anions, X^- (=N_3^-, SCN^-, OH^-, etc.), in metHrX complexes. The μ-oxo bridge mediates a high degree of antiferromagnetic coupling between the iron atoms in met- and oxyHrs ($J \sim -100$ cm^{-1}), leading to a ground spin state $S = 0$ (Dawson et al., 1972a; Maroney et al., 1986). The extent of coupling is lowered by 5–10 times upon reductions of the iron site to the [Fe(II),Fe(III)]semi-met and [Fe(II),Fe(II)]deoxy levels (Maroney et al., 1986; Reem & Solomon, 1987; Pearce et al., 1987). In the cases of semimetHrX complexes and deoxyHr this lowering has been attributed to protonation of the μ-oxo bridge with essential conservation of the remaining structural features of the iron site in metHr. The ground spin state, $S = {}^1/_2$, at the semi-met level is detectable below 30 K as a characteristic EPR signal with $g_{av} \sim 1.84$ (Muhoberac et al., 1980). The product of one-electron reduction of metHr is referred to as (semi-met)$_R$Hr, which can be distinguished from the alternative product of one-electron oxidation of deoxyHr, (semi-met)$_O$Hr, by the shape of its EPR spectrum and by its redox kinetics.

A large body of kinetic data is available for redox reactions of Hr using inorganic redox partners (Wilkins & Harrington, 1983; Nocek et al., 1984; Armstrong et al., 1985; Bradić et al., 1986). Physiological relevance was conferred on this body of data by our recent discovery of what appears to be a redox partner of Hr, namely, cytochrome b_5, in erythrocytes of two

† This research was supported by a grant from the National Institutes of Health (GM 37851 to D.M.K.).

[1] Abbreviations: Hr, hemerythrin; μ-S^{2-}metHr, μ-sulfidomethemerythrin; EDTA, ethylenediaminetetraacetate; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; MES, 2-(*N*-morpholino)ethanesulfonate; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonate; Mb, myoglobin; 15-aneN_4, 1,4,8,12-tetraazacyclopentadecane; phen, 1,10-phenanthroline; EPR, electron paramagnetic resonance; cyt b_5, cytochrome b_5; NHE, normal hydrogen electrode.

0006-2960/87/0426-8709$01.50/0

sipunculids, *Phascolopsis gouldii* and *Themiste zostericola* (Utecht & Kurtz, 1985, 1987). We have presented evidence that a function of cytochrome b_5 is to reduce metHr within the sipunculan erythrocyte. In order to provide a framework for understanding the mechanism of this reduction, we have recently constructed a scheme for reduction of the iron site in metHr by "outer-sphere" reagents (Pearce et al., 1987). According to this scheme, the iron atom closer to the outer surface of each subunit in the octamer, Fe1, is reduced upon conversion of [Fe(III),Fe(III)]metHr to [Fe(II),Fe(III)]-(semi-met)$_R$Hr. Further reduction to [Fe(II),Fe(II)]deoxyHr is then dependent upon a protein conformational change, which we propose is accompanied by intrasite electron exchange between Fe1 and Fe2 and, perhaps, protonation of the μ-oxo bridge.

Another relevant discovery in this laboratory is the redox chemistry of a sulfide derivative of the iron site in Hr, in which the μ-oxo bridge is replaced by a μ-sulfido bridge (Lukat et al., 1984; Lukat & Kurtz, 1985). This replacement appears to have a minimal effect on the surrounding protein and on the remaining structural features of the iron site. However, the [Fe(III),Fe(III)]met → [Fe(II),Fe(III)]semi-met reduction potential increases from 110 mV in native metHr to 295 mV in μ-S^{2-}metHr. Evidence from NMR and EPR spectroscopies indicates that the magnitude of antiferromagnetic coupling is lowered ~3-fold when μ-S^{2-}metHr is reduced to μ-S^{2-}semi-metHr (Maroney et al., 1986; Pearce et al., 1987).

Variations in rates of elecron transfer between two heme centers in proteins have been explained in terms of factors such as electrostatic interactions, driving force, reorganizational energy, distance, intervening medium, and orientation (Scott et al., 1985; McLendon & Miller, 1985; Peterson-Kennedy et al., 1986). However, less is known about dependence on such factors for electron transfer between a heme center and a non-heme iron center of the type found in Hr.

In the present investigation we use both metal complexes and heme proteins as redox partners of *P. gouldii* Hr and μ-S^{2-}Hr at the met and semi-met oxidation levels. The resulting kinetic data are analyzed within the framework of Marcus theory of electron-transfer reactions as applied to proteins (Wherland & Gray, 1977), and in terms of the known structural and electronic properties of the various reagents.

Experimental Procedures

Preparation of MetHr. Live worms of the species *P. gouldii* were obtained from Marine Biological Laboratory, Woods Hole, MA. OxyHr was isolated from the coelomic erythrocytes and crystallized by a standard procedure (Klotz & Klotz, 1957). The crystalline protein was stored frozen in liquid N_2. Solutions of oxyHr were prepared by dissolving the crystalline protein in 50 mM Tris–acetate, pH 8.0. MetHr was prepared by dialysis at 4 °C of solutions of oxyHr against 50 mM HEPES, pH 7.0, containing 2 mM $K_3Fe(CN)_6$. Excess oxidant was removed by extensive dialysis against the buffer of choice. Typically, ~5 mL of metHr solution was dialyzed against three to four 1-L changes of buffer over a 24-h period. Concentrations of metHr were determined by the addition of sodium azide and the use of ϵ_{446} = 3700 M^{-1} cm^{-1} (Darnall et al., 1969). All Hr concentrations are expressed in terms of binuclear iron sites. Most solutions of metHr were stored frozen in liquid N_2 and thawed prior to use.

Preparation of (Semi-met)$_R$Hr. Oxygen was removed from buffered solutions of ~0.1 mM metHr either by anaerobic dialysis or by alternate evacuation and flushing with N_2. One equivalent of $[Cr(15\text{-ane}N_4)(H_2O)_2]^{2+}$ was injected via a gas-tight syringe in an anaerobic cuvette. Completeness of reduction was judged by monitoring absorbance at 380 nm and by EPR spectroscopy (Pearce et al., 1987). Reactions were complete within 1 min at room temperature. Solutions of (semi-met)$_R$Hr were used within a few minutes of preparation.

Preparations of μ-S^{2-}metHr and μ-S^{2-}semi-metHr. These preparations are based on previously described procedures (Lukat et al., 1984; Lukat & Kurtz, 1985). All operations were carried out under N_2 at 4 °C unless otherwise noted. μ-S^{2-}metHr was prepared by dialysis of ~1 mM metHr against 3–5 mM $Na_2S{\cdot}9H_2O$ in 50 mM HEPES, pH 7.0, for ~10 h. The resulting solution of μ-S^{2-}semi-metHr was oxidized to μ-S^{2-}metHr by dialysis against buffer containing 3–4 mM $K_3Fe(CN)_6$ for several hours. The solution of μ-S^{2-}metHr was passed rapidly by centrifugation over small columns of Sephadex G-25 in 5-mL plastic syringe barrels (aerobically) followed by anaerobic dialysis against the appropriate buffer for several hours to remove any remaining $Fe(CN)_6^{3-}$. Concentrations were determined by using ϵ_{464} = 4500 M^{-1} cm^{-1} (Lukat et al., 1984). μ-S^{2-}semi-metHr was prepared by dialysis of metHr against sulfide as described above. Excess sulfide was removed either by dialysis or by passage over the small Sephadex G-25 columns, as described above. Alternatively, μ-S^{2-}semi-metHr was prepared by addition of 1 equiv of $Na_2S_2O_4$ to a solution of μ-S^{2-}metHr. No differences in kinetic behavior were observed for μ-S^{2-}semi-metHr prepared by the two methods. Concentrations of μ-S^{2-}semi-metHr were determined by use of ϵ_{500} = 1100 M^{-1} cm^{-1}. Solutions of μ-S^{2-}metHr and μ-S^{2-}semi-metHr were stored frozen in liquid N_2.

Preparation of DeoxyMb. Sperm whale Mb was obtained from Sigma Chemical Co. and passed over a column of Sephadex G-25 equilibrated with 10 mM phosphate, pH 7.0, prior to dialysis against the appropriate buffer. DeoxyMb was prepared by anaerobic titration with $Na_2S_2O_4$ immediately before use. Concentrations of $Na_2S_2O_4$ were determined by titration with solutions of $K_3Fe(CN)_6$, whose concentrations were determined by using ϵ_{420} = 1100 M^{-1} cm^{-1} (Irwin et al., 1983). Solutions of deoxyMb prepared in this manner were used within a few minutes of preparation to avoid autoxidation. Concentrations of deoxyMb were obtained spectrophotometrically by using ϵ_{560} = 1.2 × 10^4 M^{-1} cm^{-1} (Bradič et al., 1979).

Preparation of Reduced P. gouldii Cytochrome b_5. Cytochrome b_5 was isolated from erythrocytes of *P. gouldii* as described by Utecht and Kurtz (1987). Approximately 1 μM solutions of cytochrome b_5 were reduced either by anaerobic titration with $Na_2S_2O_4$ or by brief irradiation with a 500-W projector lamp in the presence of ~1 mM EDTA and ~0.1 μM riboflavin (Massey & Hemmerich, 1977). Concentrations of cytochrome b_5 were determined prior to reduction by using ϵ_{412} = 1.15 × 10^5 M^{-1} cm^{-1}.

Preparation of Inorganic Reagents. Solutions of Cr^{2+}(aq) were prepared by oxidation of Cr metal as previously described (Pearce et al., 1987). The resulting solutions were diluted with 0.1 M cacodylate, pH 7.0, containing 50 mM $NaClO_4$. Concentrations of Cr^{2+} were determined by titration with sodium permanganate by using ϵ_{545} = 2340 M^{-1} cm^{-1} (Dawson et al., 1972b). Solutions of $[Cr(15\text{-ane}N_4)(H_2O)_2]^{2+}$ were prepared by anaerobic addition of Cr^{2+}(aq) to solutions of 15-aneN_4 obtained from Strem Chemical Co. Concentrations of $[Cr(15\text{-ane}N_4)(H_2O)_2]^{2+}$ were determined by using ϵ_{540} = 36.5 M^{-1} cm^{-1} (Adzamli et al., 1984). $Fe(EDTA)^{2-}$ and $[Co(phen)_3]Cl_3$ were prepared and their concentrations determined by previously described methods (Wherland et al., 1975; Segal & Sykes, 1978).

Determinations of Isoelectric Points. The isoelectric pHs (p*I*'s) of metHr and μ-S^{2-}metHr were determined in parallel by using an LKB Bromma Model 2117 Multiphor isoelectric focusing apparatus. The ampholite solution was a 50–50 mixture of LKB ampholine pH 3–10 and pH 5–8. The polyacrylamide gels were 2.6% cross-linked.

Kinetic Measurements. For reactions of Hr and μ-S^{2-}Hr with inorganic reagents, 0.1 mM protein and a 10-fold or larger molar excess of inorganic reagent were used. The exception was oxidation by $K_3Fe(CN)_6$, where a 0.05 mM concentration of protein was used. Buffers at 50 mM were MES, pH 6.3; HEPES, pH 7.0; or HEPPS, pH 8.2. All protein solutions contained 150 mM Na_2SO_4 except for those to be mixed with Cr^{2+}/cacodylate, which instead contained 50 mM $NaClO_4$. Concentrations of Cr^{2+}/cacodylate in protein solutions were kept at or below 2 mM, since higher concentrations caused precipitation of Hr. All reactions were performed under N_2 which had been passed through chromous scrubbing towers to remove traces of O_2. Reactions were initiated by using either conventional or stopped-flow mixing techniques. The stopped-flow device has been described previously (Pearce et al., 1987). Conventional mixing was carried out in a 1 cm path semimicrocuvette, with injection of reagents through gas-tight syringes. These reactions were monitored by using a Perkin-Elmer Model 554 spectrophotometer. Reactions of metHr and $(semi\text{-}met)_RHr$ were monitored between 400 and 320 nm; reactions of μ-S^{2-}metHr and μ-S^{2-}semi-metHr were monitored at 464 nm, the λ_{max} for μ-S^{2-}metHr. Total reaction volumes were ~1.0 mL for conventional mixing. Constant temperature (±0.1 °C) was maintained by a Brinkman constant temperature bath connected to a thermostated cell holder or mixing chamber. Absorbance vs time data were either fit to exponential functions by using the nonlinear least-squares program EXPSUM (provided by Dr. J. H. Espenson) or plotted as $\ln (A_t - A_\infty)$ vs time, in which case least-squares analysis was used. Reactions were followed to at least 80% completion. Rate constants reported are the average of 3–5 replicate determinations.

For measurements in D_2O ~1 mL of metHr was dialyzed against ~100 mL of buffered D_2O for ~24 h at 4 °C. Buffered D_2O solutions were adjusted to the required pD (=pH meter reading + 0.4). Reactions were carried out with $Fe(EDTA)^{2-}$ and $[Co(phen)_3]Cl_3$ that had been prepared in buffered D_2O solutions.

All protein–protein reactions were performed anaerobically by conventional mixing and monitoring as described above. Concentrations of deoxyMb for all reactions were between and 10 and 30 μM. Usually a 10-fold or larger molar excess of either metHr or μ-S^{2-}metHr (calculated on the basis of binuclear iron sites) over deoxyMb was used. Oxidations of deoxyMb were monitored at either 560 or 430 nm. Oxidations of *P. gouldii* cytochrome b_5 were monitored at 422 nm (the λ_{max} for reduced cytochrome b_5). The concentration of cytochrome b_5 was 1 μM in all reactions. Concentrations of metHr and μ-S^{2-}metHr ranged from 10 to 1000 μM. Buffers were either those listed above for reactions with metal complexes or 10 mM phosphate, pH 6.5–7.5. Ionic strengths were adjusted with Na_2SO_4. Reactions were followed to at least 85% completion. Absorbance vs time data were analyzed as described above.

For EPR spectroscopy, samples were prepared and spectra obtained as previously described (Pearce et al., 1987).

RESULTS

An identical p*I* of 7.8 was measured for both metHr and μ-S^{2-}metHr. This isoelectric point is in good agreement with the value of ~8 previously measured for metHr (Keresztes-Nagy & Klotz, 1965).

Table I: Second-Order Rate Constants for Reductions of MetHr and μ-S^{2-}metHr by Various Reagents at 25 °C[a]

reductant [E° (mV) vs NHE]	k_{12}(met) (M^{-1} s^{-1})	k_{12}(μ-S^{2-}met) (M^{-1} s^{-1})	k_{12}(μ-S^{2-}met)/k_{12}(met)
$Fe(EDTA)^{2-}$ [b] (120[d])	5.4 (±0.1)	350 (±3)	65
Cr^{2+}/cacodylate[c] (−410)	42 (±4)	1062 (±90)	25
$[Cr(15\text{-}aneN_4)]^{2+}$ [b] (−580[e])	950 (±90)	32000 (±4000)	34
deoxyMb[b] (50[f])	1.2 (±0.3)	38 (±4)	31
P. gouldii cyt b_5[g] (7[h])	160 (±10)	8900 (±600)	56

[a] Data for $[Cr(15\text{-}aneN_4)]^{2+}$ were obtained at 20 °C. [b] 50 mM MES, pH 6.3, and 0.15 M Na_2SO_4. [c] 0.1 M cacodylate, pH 7.0, and 50 mM $NaClO_4$. [d] Wherland & Gray, 1977. [e] Samuels & Espenson, 1979. [f] Brunori et al., 1971. [g] 10 mM phosphate, pH 7.5, and 0.15 M Na_2SO_4. [h] Utecht & Kurtz, 1987.

Table II: Activation Parameters for Reductions of MetHr and μ-S^{2-}metHr by Various Reagents

reductant	$\Delta H^\ddagger$ (kJ/mol)	$\Delta S^\ddagger$ [J/(mol·K)]
	μ-S^{2-}metHr	
$Fe(EDTA)^{2-}$[a]	23.0 (±0.2)	−72 (±1)
Cr^{2+}/cacodylate[b]	20 (±5)	−72 (±17)
	MetHr	
$Fe(EDTA)^{2-}$[a]	28.2 (±0.3)	−137 (±2)
Cr^{2+}/cacodylate[b]	27.3 (±0.3)	−122 (±3)
P. gouldii cyt b_5[c]	35.7 (±0.1)	−36 (±8)

[a] 50 mM MES, pH 6.3, and 0.15 M Na_2SO_4. [b] 0.1 M cacodylate, pH 7.0, and 50 mM $NaClO_4$. [c] 10 mM phosphate, pH 7.5.

Redox Kinetics of Hr and μ-S^{2-}Hr with Metal Complexes. (*A*) *Reductions.* In Table I are reported the second-order rate constants for reductions of metHr and μ-S^{2-}metHr to the semi-met oxidation level by $Fe(EDTA)^{2-}$, Cr^{2+}/cacodylate, and $[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$. In all cases the observed first-order rate constants are linearly dependent on reductant concentrations in the range of 1–4 mM. These latter data are available as supplementary material (see paragraph at end of paper regarding supplementary material). Due to their relatively negative reduction potentials (Table I), Cr^{2+}/cacodylate and $[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$ can reduce metHr to deoxyHr. However, we have shown previously (Pearce et al., 1987) that, for these reagents, the first step, namely, reduction of metHr to $(semi\text{-}met)_RHr$, is much faster and, therefore, easily separated from subsequent reduction to deoxyHr. With the weakest reducing agent, $Fe(EDTA)^{2-}$, in the range of 1–4 mM, no reduction beyond the semi-met level was observed. This result was confirmed by subsequent addition of azide, which resulted in the absorption spectrum of semi-metHrN_3 (Wilkins & Harrington, 1983). Consistent with previous observations using $Na_2S_2O_4$ (Lukat & Kurtz, 1985), no further reduction of μ-S^{2-}semi-metHr was observed with any of the reagents listed above. The data in Table I show that the rates of reduction by Cr^{2+}/cacodylate, $[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$, and $Fe(EDTA)^{2-}$ are 25–65 times faster for μ-S^{2-}metHr than for metHr. The pH dependences of the second-order rate constants for reductions of metHr and μ-S^{2-}metHr by $Fe(EDTA)^{2-}$ in the presence and absence of the allosteric effector ClO_4^- are shown in Figure 1. Activation parameters for the reductions of metHr and μ-S^{2-}metHr by Cr^{2+}/cacodylate and $Fe(EDTA)^{2-}$ are listed in Table II. These values were determined from Eyring plots, data for which are available as supplementary material. We have previously reported (Pearce et al., 1987) that the rate of reduction of metHr to $(semi\text{-}met)_RHr$ by $Fe(EDTA)^{2-}$ shows no D_2O effect.

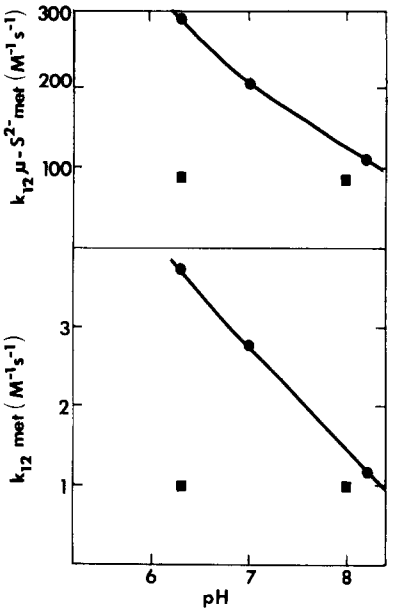


FIGURE 1: Dependence upon pH of second-order rate constants for reductions of metHr (bottom) and μ-S^{2-}metHr (top) by $Fe(EDTA)^{2-}$. Squares represent reactions in the presence of 50 mM $NaClO_4$. Conditions: 0.1 mM Hr in 50 mM MES, HEPES, or HEPPS at pH 6.3, 7.0, or 8.2, respectively, and 0.15 M Na_2SO_4, 20 °C. Solid curves do not represent any theoretical fit of the data.

Table III: Second-Order Rate Constants for Oxidations of (Semi-met)$_R$Hr and μ-S^{2-}semi-metHr by $[Co(phen)_3]Cl_3$ and $K_3Fe(CN)_6$[a]

oxidant	k_{12}[(semi-met)$_R$] (M^{-1} s^{-1})	k_{12}(μ-S^{2-}semi-met) (M^{-1} s^{-1})	k_{12}[(semi-met)$_R$]/k_{12}(μ-S^{2-}semi-met)
$[Co(phen)_3]Cl_3$	1.3 (±0.2)	8.2 (±0.4)	0.12
$K_3Fe(CN)_6$	[1.6 (±0.3)] × 10^4	326 (±10)	49

[a] 50 mM HEPPS, pH 8.2, and 0.15 M Na_2SO_4; 25 °C.

(*B*) *Oxidations.* The kinetics of oxidation of *P. gouldii* (semi-met)$_R$Hr by $K_3Fe(CN)_6$ have been previously studied by Wilkins and co-workers (Bradić et al., 1980; Harrington & Wilkins, 1983). In the present study the oxidation of μ-S^{2-}semi-metHr and (semi-met)$_R$Hr by $[Co(phen)_3]Cl_3$ was carried out as well as the oxidation of μ-S^{2-}semi-metHr by $K_3Fe(CN)_6$. Since a different method of preparation of (semi-met)$_R$Hr was used in the present study, its kinetics of oxidation by $K_3Fe(CN)_6$ were reexamined. In Table III the second-order rate constants for the above reactions are shown. The linear dependences of observed first-order rate constants on concentrations of oxidants are shown in Figure 2. Addition of Cl^- to 100 mM does not appreciably affect the rates of oxidation of (semi-met)$_R$Hr or μ-S^{2-}semi-metHr by $[Co(phen)_3]Cl_3$. For oxidations by $[Co(phen)_3]Cl_3$ at pH 8.2 activation parameters determined from Eyring plots (cf. supplementary material) are as follows: for μ-S^{2-}semi-metHr, $\Delta H^{\ddagger}$ = 30.0 (±0.2) kJ/mol and $\Delta S^{\ddagger}$ = −80 (±1) J/mol; J/mol; and for (semi-met)$_R$Hr, $\Delta H^{\ddagger}$ = 76 (±1) kJ/mol and $\Delta S^{\ddagger}$ = 59 (±3) J/mol. The oxidation of (semi-met)$_R$Hr by $[Co(phen)_3]Cl_3$ was also carried out in ~95% D_2O at 25 °C and pD 8.2. The second-order rate constant obtained in D_2O, 1.7 (±0.2) M^{-1} s^{-1}, is, within experimental error, identical with that obtained in H_2O (Table III).

Reduction Kinetics of MetHr and μ-S^{2-}metHr with Heme Proteins. (*A*) *DeoxyMb.* The kinetics of reduction of metHr by deoxyMb have previously been described by Bradić et al. (1979). In order to ensure a valid comparison with the reaction between μ-S^{2-}metHr and deoxyMb, we have reexamined the kinetics with metHr. The resulting second-order rate constants

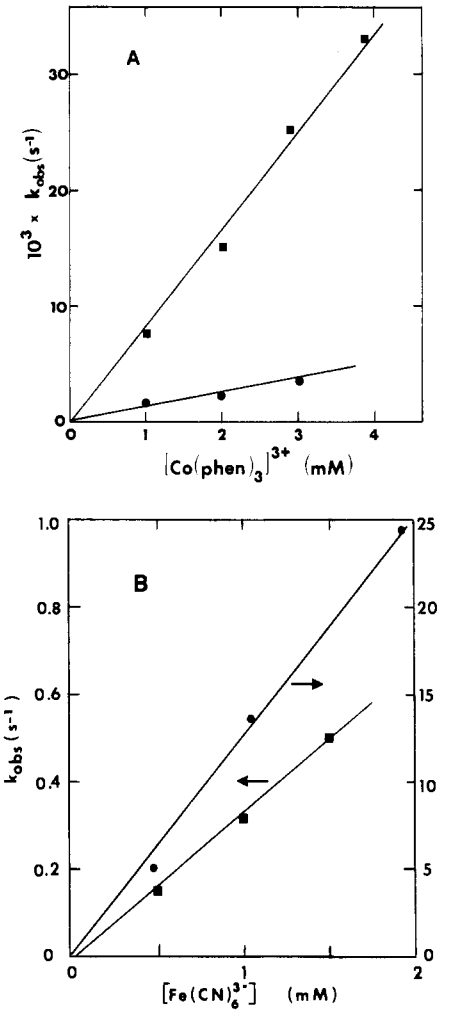


FIGURE 2: Dependence of observed first-order rate constants for oxidations of (semi-met)$_R$Hr (circles) or μ-S^{2-}semi-metHr (squares) on concentrations of (A) $[Co(phen)_3]Cl_3$ or (B) $K_3Fe(CN)_6$. Conditions: 50 mM HEPPS, pH 8.2, and 0.15 M Na_2SO_4, 25 °C; 0.1 mM Hr for (A) and 0.05 mM Hr for (B).

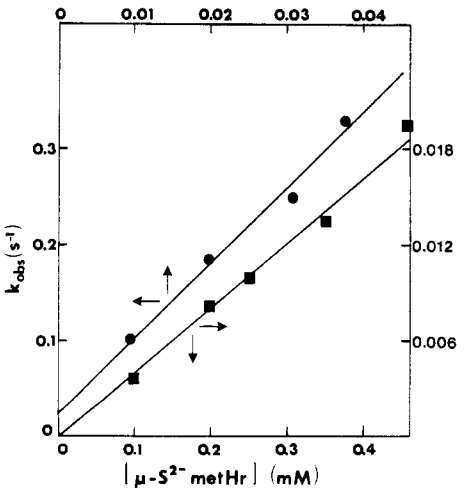


FIGURE 3: Dependence of observed first-order rate constants for oxidation of deoxyMb (squares, lower and right axes) or *P. gouldii* cytochrome b_5 (circles, upper and left axes) on the concentration of μ-S^{2-}metHr. Conditions: 30 μM deoxyMb in 50 mM MES, pH 6.3, or 1 μM cytochrome b_5 in 10 mM phosphate, pH 7.5. All solutions contained 0.15 M Na_2SO_4, and the temperature was 25 °C.

for both reactions are listed in Table I, and the dependence of k_{obsd} on concentration of μ-S^{2-}metHr is shown in Figure 3. Isosbestic points at ~520 and ~610 nm and uniphasic absorbance changes were maintained during oxidations of de-

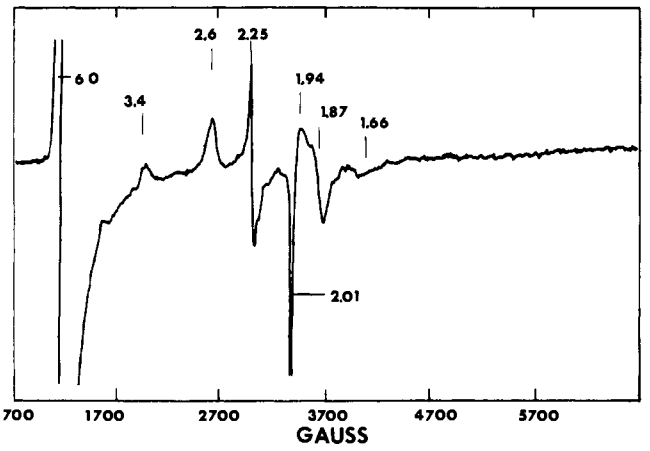


FIGURE 4: EPR spectrum obtained during the oxidation of 0.2 mM deoxyMb by 0.2 mM metHr. Conditions: 50 mM HEPES, pH 7.0, and 0.15 M Na_2SO_4, 25 °C. A 0.1-mL sample of the reaction solution was frozen in liquid N_2 11 min after mixing. EPR parameters are as follows: T, 4 K; power, 0.2 mW; frequency, 9.46 GHz; modulation, 16 G at 100 kHz.

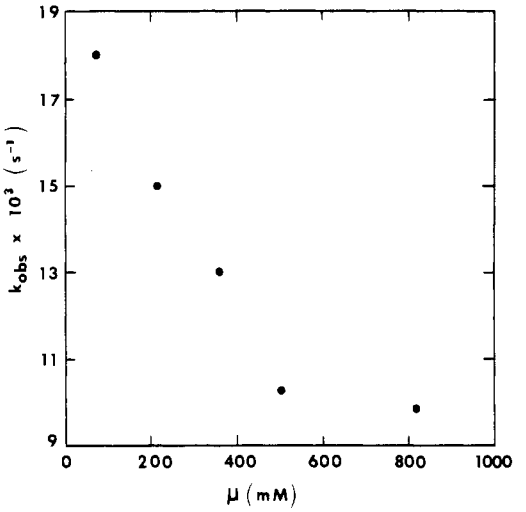


FIGURE 5: Effect of ionic strength on observed first-order rate constant for oxidation of reduced *P. gouldii* cytochrome b_5 by metHr. Conditions: 1.2 μM cytochrome b_5, 61 μM metHr, and 10 mM phosphate, pH 7.5, 25 °C. Ionic strength was adjusted by addition of Na_2SO_4.

oxyMb, which is very similar to the behavior previously observed by Bradić et al. (1979). These authors reported deoxyHr as the ultimate product of the reaction with metHr. We ascertained production of metMb by the change in λ_{max} of the Soret band from 430 and 411 nm. As with the inorganic reagents, no reduction of μ-S^{2-}Hr beyond the semi-met level was observed. Previous work (Wilkins & Harrington, 1983) suggests that (semi-met)$_R$Hr reacts more rapidly than metHr with deoxyMb. We used EPR spectroscopy to show that (semi-met)$_R$Hr is produced, at least as a steady-state intermediate, in the reaction of metHr with deoxyMb. As shown in Figure 4, a 1:1 mol:mol solution of the two proteins frozen after 11-min reaction at 25 °C exhibits an EPR spectrum with g values at 1.94, 1.87, and 1.66 characteristic of (semi-met)$_R$Hr. The remaining features in the spectrum are due to metMb. EPR spectra obtained at later reaction times (up to 225 min) also show the features of (semi-met)$_R$Hr.[2] Thus, for the reactions of both metHr and μ-S^{2-}metHr with deoxyMb, the rate constants listed in Table I apparently represent reduction of the met to the semi-met oxidation level.

(*B*) *P. gouldii Cytochrome* b_5. Table I also lists second-order rate constants for reactions of reduced *P. gouldii* cytochrome b_5 with metHr and μ-S^{2-}metHr. The linear dependence of k_{obsd} on concentration of μ-S^{2-}metHr is shown in Figure 3. The linear dependence of k_{obsd} on concentration of *P. gouldii* cytochrome b_5 is shown elsewhere (Utecht & Kurtz, 1987). The nonzero intercept in Figure 3 is due to autoxidation of cytochrome b_5 and is also observed in the reaction with metHr. This autoxidation makes reduction of Hr beyond the semi-met level impossible in the absence of additional constituents. The rate constants listed in Table I and shown in Figure 5 do not include the contribution due to autoxidation of cytochrome b_5. Thus, as with the metal complexes, these second-order rate constants represent reduction of the met to the semi-met oxidation level. For both heme proteins, the ratio $k_{12}(\mu\text{-}S^{2-}\text{metHr})/k_{12}(\text{metHr})$ is within the range of values listed in Table I for the metal complexes. The activation parameters for the oxidation of *P. gouldii* cytochrome b_5 by metHr calculated from an Eyring plot (cf. supplementary material) are included in Table II. The value of $\Delta H^{\ddagger}$ is similar to that obtained for reductions by the metal complexes, while the value of $\Delta S^{\ddagger}$ is less negative for the protein–protein reaction.

The second-order rate constants for the oxidation of *P. gouldii* cytochrome b_5 by metHr at 25 °C and 150 mM Na_2SO_4 are pH dependent: 490 M^{-1} s^{-1} at pH 6.5, 280 M^{-1} s^{-1} at pH 7.0, and 160 M^{-1} s^{-1} at pH 7.5. However, confirming previous results (Bradić et al., 1979), we find little variation with pH of the second-order rate constant for the oxidation of deoxyMb by metHr.

The ionic strength dependence of the rate of oxidation of *P. gouldii* cytochrome b_5 by metHr at pH 7.5 is shown in Figure 5. As can be seen, the rate decreases as the ionic strength increases. We find the same trend, though less pronounced, for the deoxyMb–metHr reaction, where the second-order rate constant at 25 °C and pH 6.3 decreases from 1.2 M^{-1} s^{-1} at 0.15 M Na_2SO_4 to 0.7 M^{-1} s^{-1} at 0.5 M Na_2SO_4.

Reduction of P. gouldii Cytochrome b_5 *by* $Fe(EDTA)^{2-}$. Since published values are available for the kinetics of reduction of beef liver cytochrome b_5 by $Fe(EDTA)^{2-}$ (Reid & Mauk, 1982), we carried out the reduction of *P. gouldii* cytochrome b_5 by $Fe(EDTA)^{2-}$ in order to provide a comparison of redox reactivity. At pH 7.0, μ = 0.2 (phosphate), and 25 °C, the second-order rate constant for the reduction of 1 μM *P. gouldii* cytochrome b_5 by a 1000-fold or greater molar excess of $Fe(EDTA)^{2-}$ was found to be 256 ± 20 M^{-1} s^{-1}. With less than 1 mM $Fe(EDTA)^{2-}$ the reaction did not go to completion. The published value for the second-order rate constant under the same conditions for reduction of beef liver cytochrome b_5 by $Fe(EDTA)^{2-}$ is 186 M^{-1} s^{-1} (Reid & Mauk, 1982). In this case, concentrations of $Fe(EDTA)^{2-}$ <0.5 mM led to incomplete reduction. Thus, according to this comparison, the redox reactivities of the two cytocromes b_5 are quite similar.

Discussion

Several previous studies have examined the effects of changes in the reduction potentials of small-molecule reagents on the rates of electron-transfer reactions of metal centers in proteins, including the iron site in Hr (Harrington & Wilkins, 1983, Bradić et al., 1986). However, we are unaware of any previous examinations of the effects on these rates of changing the potential of the metal center *within* a non-heme protein. The availability of metHr and μ-S^{2-}metHr, whose met → semi-met reduction potentials differ by ~200 mV, therefore,

[2] Overlap of the metMb and (semi-met)$_R$Hr EPR signals prevented us from obtaining spin concentrations.

provides a unique opportunity to examine such effects. In order to examine the generality of these effects, we have used as reducing agents a set of metal complexes and heme proteins (listed in Table I) that possess a range of reduction potentials, net charges, and mechanistic possibilities.

As shown in Figure 1, the rates of reductions of metHr and μ-S^{2-}metHr by $Fe(EDTA)^{2-}$ exhibit parallel dependences on pH and on the presence of the allosteric effector, ClO_4^-. $Fe(EDTA)^{2-}$ does not have an ionizable functional group with a pK_a in the pH range examined in this study, and in addition, the variation with pH is abolished by ClO_4^-. Therefore, the variations in rates shown in Figure 1 are most likely due either to ionization of a functional group on the protein or to a pH-dependent conformational change of the protein (Reid & Mauk, 1982; Reid et al., 1984). For reductions of *T. zostericola* met- to $(semi\text{-}met)_RHr$ by a series of positively charged cobalt complexes, Armstrong et al. (1985) observed pH-dependent rate constants having the same trend and similar percentage changes as those of Figure 1. Their analysis of the data assumed a deprotonation–protonation equilibrium of metHr and yielded a pK_a of 7.6. The limited number of points in Figure 1 preclude such an analysis of our data.

The five-coordinate iron atom, Fe2, in metHr is the one that binds OH^- at pHs $\gtrsim$ 8 (Shiemke et al., 1986). Previous results (Lukat et al., 1984; Lukat & Kurtz, 1985) strongly suggest that OH^- *does not* bind to Fe2 (or Fe1) in either μ-S^{2-}metHr or μ-S^{2-}semi-metHr, yet the pH dependence of the reduction kinetics of μ-S^{2-}metHr parallels that of metHr (Figure 1). Thus, we conclude that the parallel pH dependences are *not* due to binding of OH^- to Fe2. Further insight into this point can be gained from the kinetics of reductions of metHr and μ-S^{2-}metHr by $Fe(EDTA)^{2-}$ in the presence of ClO_4^- (Figure 1). X-ray crystallographic studies of metHr show that ClO_4^- binds to the outer surface of the Hr octamer and that this binding induces changes in electron density near the five-coordinate iron atom, Fe2 (Stenkamp et al., 1983, 1984). These changes are manifested in solution between pHs 7 and 9 as absorbance changes, which have been attributed to stabilization of an "acid" form of metHr (Bradić & Wilkins, 1983). Perchlorate is also known to increase the stabilities of both μ-S^{2-}metHr and μ-S^{2-}semi-metHr (Lukat et al., 1984, 1985). However, stabilization of the "acid" form by ClO_4^- is not reflected in the data of Figure 1. Values of the rate constants for reductions of metHr and of μ-S^{2-}metHr by $Fe(EDTA)^{2-}$ near pH 8 are very similar in the presence or absence of ClO_4^-. The rate constants in the presence of ClO_4^- remain virtually unchanged when the pH is lowered to 6.3. Binding of ClO_4^- appears to "lock" both metHr and μ-S^{2-}metHr into a protein conformation that is relatively unaffected by pH. Thus, from the data of Figure 1, we conclude that differences between rates of reduction of metHr and μ-S^{2-}metHr, at least in the pH range of 6–8, are due almost totally to intrinsic differences in the redox properties of the iron sites, rather than to differences in water or hydroxide binding to the iron atoms or to differences in the protein matrix. This conclusion is consistent with our finding an identical value, 7.8, for the isoelectric pHs of metHr and μ-S^{2-}metHr. We also conclude, consistent with our previous proposal (Pearce et al., 1987), that the six-coordinate iron atom, Fe1, is reduced during transformations of both metHr and μ-S^{2-}metHr to the semi-met level.

The simple Marcus relation for outer-sphere electron-transfer reactions is

$$k_{12} = (k_{11}k_{22}K_{12})^{1/2} \quad (1)$$

where k_{12} is the cross-reaction rate constant (i.e., those listed in Tables I and III), K_{12} is the equilibrium constant for the cross-reaction ($=e^{38.94\Delta E}$), and k_{11} and k_{22} are the electron self-exchange rate constants for the two reagents (Wherland & Gray, 1977). From this relation it is obvious that the 185-mV increase in met → semi-met reduction potential for μ-S^{2-}metHr over metHr should lead to increases in rates of reduction. For purposes of calculating relative rates, we assume, on the basis of the preceding discussion, that the self-exchange rate constants, k_{11}, for metHr and μ-S^{2-}metHr are identical. Then, the ratio

$$k_{12}(\mu\text{-}S^{2-}met)/k_{12}(met) = (e^{38.94\Delta E})^{1/2} \simeq 40$$

The experimentally determined values of this ratio listed in Table I are in good agreement with this calculation over a fairly large range of net charge on the metal complex, driving force, and mechanistic differences.[3] Note that in the case of Cr^{2+}/cacodylate, agreement with the calculated ratio is good even in the presence of the allosteric effector, ClO_4^-. The activation enthalpies for the reactions of $Fe(EDTA)^{2-}$ and Cr^{2+}/cacodylate with both metHr and μ-S^{2-}metHr (Table II) are all very similar, although somewhat lower values are obtained for the reductions of μ-S^{2-}metHr. The differences in activation entropies between metHr and μ-S^{2-}metHr apparently reflect the differences in reorganization of the two iron sites necessary to reach the transition states. We believe that these differences in activation parameters do not reflect large mechanistic differences, such as a reversal of the iron atom being reduced, for reduction of μ-S^{2-}metHr vs metHr. The agreement between calculated and observed ratios, coupled with the lack of D_2O effects on the rates of either reduction of metHr or oxidation of $(semi\text{-}met)_RHr$, supports our recent suggestion (Pearce et al., 1987) that the μ-oxo bridge may not be protonated in $(semi\text{-}met)_RHr$. Resonance Raman studies have indicated no protonation of the μ-sulfido bridge in μ-S^{2-}semi-metHr (Freier et al., 1979).

In the case of oxidations, once again assuming an identical value of k_{11}, the calculated ratio

$$k_{12}[(semi\text{-}met)_R]/k_{12}(\mu\text{-}S^{2-}met) \simeq 40$$

As can be seen from the data in Table III, this calculation is borne out experimentally when $K_3Fe(CN)_6$ is used as oxidant. Ferricyanide is known to bind weakly to both metHr (Bradić et al., 1980) and μ-S^{2-}metHr.[4] The agreement with the calculated ratio of rate constants suggests that, if ferricyanide accepts an electron while at its binding sites, these sites must be very similar for $(semi\text{-}met)_RHr$ and μ-S^{2-}semi-metHr.

Our value of k_{12} for oxidation of $(semi\text{-}met)_RHr$ by $K_3Fe(CN)_6$ is ~50-fold higher than that reported under similar conditions by Bradić et al. (1980). We attribute the apparent discrepancy to the different methods of preparation of $(semi\text{-}met)_RHr$ in the two cases. We have documented that reduction of metHr by $[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$ results in quantitative production of $(semi\text{-}met)_RHr$ and that no Cr is bound to $(semi\text{-}met)_RHr$, when produced in this fashion (Pearce et al., 1987). The value of k_{12} reported by Bradić et al. (1980) refers to $(semi\text{-}met)_RHr$ produced by irradiation of solutions containing metHr in one of the driving syringes of a stopped-flow apparatus.

[3] We have found that Cr(III) binds to the product, $(semi\text{-}met)_RHr$, in a 1:1 mole ratio when Cr^{2+}(aq) is used as reducing agent, whereas no binding of Cr is observed when $[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$ is used (Pearce et al., 1987).

[4] L. L. Pearce and D. M. Kurtz, Jr., unpublished results. While we have not examined an extensive concentration range, we have noted no curvature in plots of k_{obsd} vs $[K_3Fe(CN)_6]$.

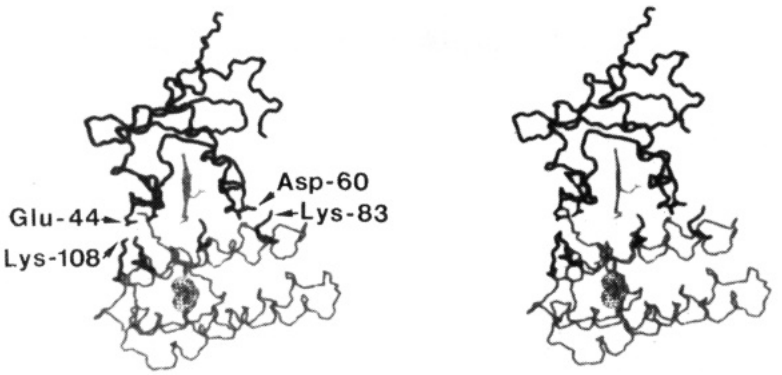


FIGURE 6: Stereo diagram of docked beef liver cytochrome b_5 (top) and *T. dyscritum* Hr subunit (bottom) structures. α-Carbon backbones are shown along with side chains of residues proposed to be involved in salt-bridge formation (see text). Viewing direction of the Hr subunit is approximately perpendicular to the axes of its four helical regions, with the C and D helices on top. These two helices lie on the outer surface of each subunit in the Hr octamer (Sieker et al., 1982). All salt-bridged residues enumerated in the text are shown; for clarity only the left- and rightmost salt-bridged pairs are labeled. The heme in cytochrome b_5 is oriented approximately edge-on. The proposed heme 6-propionate–Lys-75 salt bridge lies approximately at the center in this view of the complex. The Fe–O–Fe unit of the Hr subunit is shown as three overlapping spheres with the Fe1–Fe2 axis (cf. structure in the introduction) approximately vertical and Fe1 on top. The imidazole side chain of His-101 lies directly above Fe1 and is oriented edge-on.

The experimentally determined ratio of rate constants for oxidations of metHr and μ-S^{2-}metHr using $[Co(phen)_3]Cl_3$ (Table III) is not in agreement with the calculated ratio. The activation parameters for these oxidations (vide supra) indicate that different mechanisms are operating in the two cases. The lack of a D_2O effect on the rate of oxidation of $(semi\text{-}met)_R$Hr by $[Co(phen)_3]^{3+}$ indicates that a protonated μ-oxo bridge vs an unprotonated μ-sulfido bridge is not the reason for the discrepancy. Wherland and Gray (1977) have pointed out that the hydrophobic, bladelike phen ligands of $[Co(phen)_3]^{3+}$ are expected to encourage both penetration of protein surfaces and π interactions with aromatic amino acid side chains. In the case of Hr, the penetration may be sufficient to detect differences in arrangements of residues in the vicinity of the iron site caused by replacement of bridging oxide by sulfide. A group of four interleaved aromatic residues, including the Fe1 ligand residue His-101 is known to lie between two helical regions of the metHr subunit and to block access to the iron site from the outer surface. Phe-55 is also quite close to the bridging oxo ion and is in van der Waals contact with the Fe1 ligand residue His-77 (Stenkamp et al., 1985; Sieker et al., 1982). Inspection of the structure using computer graphics shows that the larger van der Waals radius of sulfur vs oxygen and longer Fe–S vs Fe–O bond distances (Lukat et al., 1984) make it likely that one or more of the aforementioned residues must move in order to avoid van der Waals overlap with bridging sulfide.

The experimentally determined values of $k_{12}(\mu\text{-}S^{2-}met)/k_{12}(met)$ listed in Table I show that use of either deoxyMb or *P. gouldii* cytochrome b_5 as reducing agents of metHr and μ-S^{2-}metHr also results in very good agreement with the calculated ratio of 40. Thus, the simple Marcus relation is obeyed as a function of reduction potential of the iron site in Hr, with either metal complexes or heme proteins as reducing agents. Furthermore, the agreement is good whether or not electron transfer is the primary function of the heme protein.

The kinetics of reduction of *P. gouldii* erythrocyte cytochrome b_5 by $Fe(EDTA)^{2-}$ determined in the present study are very similar to those of beef liver cytochrome b_5. We have previously demonstrated that the physical properties of these two cytochromes b_5 are also very similar (Utecht & Kurtz, 1985, 1987). The rate of oxidation of *P. gouldii* cytochrome b_5 by *P. gouldii* metHr decreases as the ionic strength increases (Figure 5). This result suggests that electrostatic interactions may facilitate the formation of a protein–protein electron-transfer complex. Using computer graphics, we have found that the structures of the *Themiste dyscritum* octameric metHr subunit and beef liver cytochrome b_5 can be "docked" such that residues Lys-75, Lys-83, Lys-103, and Lys-108 on the outer surface of Hr form salt bridges (having intermolecular N---O distances of 2.8–2.9 Å) with heme 6-propionate, Asp-60, Glu-48, and Glu-44, respectively, on cytochrome b_5.[5] The structure of this docked complex is shown in Figure 6. With the exception of the salt bridges, no intermolecular van der Waals contacts of less than 3 Å are present in the docked structures. This docking also results in coplanar alignment of the heme in cytochrome b_5 with the imidazole ring of the Fe1 ligand residue His-101 in Hr. The closest heme edge–imidazole edge distance is <9 Å in this hypothetical complex. With the exception of Lys-83, all of the residues on *T. dyscritum* Hr proposed to be involved in salt bridge formation are conserved in *P. gouldii* Hr (Sanders-Loehr & Loehr, 1981). We found that Lys-88 in *P. gouldii* Hr, when superimposed on the *T. dyscritum* structure, can replace Lys-83 in a salt bridge linkage to Asp-60 on cytochrome b_5 with no other alteration in the structure described above. The residues on cytochrome b_5 cited above are those proposed to be involved in formations of electron-transfer complexes with cytochrome *c* and with hemoglobin (Mauk et al., 1986; Poulos & Mauk, 1983). Although we have not yet determined the amino acid sequence of *P. gouldii* cytochrome b_5, the aforementioned carboxylate residues are conserved in the sequences of seven other cytochromes b_5, including that from bovine erythrocyte [Runnegar (1984) and references cited therein; Slaughter et al., 1982].

Recently, intermolecular interactions between Mb and cytochrome b_5 have been demonstrated by NMR (Livingston et al., 1985; McLachlan et al., 1986). Lysine residues on Mb were proposed to serve as contact points for formation of an electron-transfer complex with cytochrome b_5 very similar to that proposed above for cytochrome b_5–Hr. If similar intermolecular interactions occur in these two electron-transfer complexes, we might expect agreement with the calculated ratio $k_{12}(cyt\ b_5\text{–}Mb)/k_{12}(cyt\ b_5\text{–}Hr)$ using the Marcus

[5] An Evans & Sutherland Picture System 300 equipped with the software package MOGLI was used. The criteria for appropriate docking of Hr and cytochrome b_5 structures were those used by Poulos and Mauk (1983). Initial atomic coordinates used were those deposited with the Brookhaven Protein Data Bank.

analysis. In order to calculate this ratio, we need not only the appropriate reduction potentials (Table I) s^{-1}) and Hr (10^{-3} M^{-1} s^{-1}) yields an estimate of the ratio $[k_{11}(Mb)/k_{11}(Hr)]^{1/2}$. For this estimate we can use values of k_{11}^{corr}, the electrostatics-corrected self-exchange rate constants at $\mu = 0.1$, pH 7, and 25 °C (Wherland & Gray, 1977). These values can be derived from rate constants for reductions of the proteins by $Fe(EDTA)^{2-}$. For Mb, the published value of k_{11}^{corr} is 0.126 (Mauk & Gray, 1979). For Hr, we use the value of 5.4 M^{-1} s^{-1} (Table I) and the methods of Wherland and Gray (1977) to calculate k_{11}^{corr} ($\mu = 0.1$ M, pH 7, 25 °C) = 5×10^{-4} M^{-1} s^{-1}. While they may not be accurate in the absolute sense, these values of k_{11}^{corr} are one measure of the relative values of the self-exchange rate constants for Mb and Hr. Thus, on the basis of reduction potentials and reactivities with $Fe(EDTA)^{2-}$, we calculate the ratio

$$k_{12}(\text{cyt } b_5\text{–Mb})/k_{12}(\text{cyt } b_5\text{–Hr}) = (e^{38.94\Delta E})^{1/2}(0.126/0.0005)^{1/2} = 5$$

Alternatively, using the values of k_{11} reported by Bradić et al. (1986) for Mb (1 M^{-1} s^{-1}) and Hr (10^{-3} M^{-1} s^{-1}) yields a calculated value of the ratio k_{12}(cyt *b*–Mb)/k_{12}(cyt b_5–Hr) = 10. The most recent published value of k_{12}(cyt b_5–Mb) is 820 M^{-1} s^{-1} at pH 6.8 (Livingston et al., 1985). The value of k_{12}(cyt b_5–Hr) determined in the present study at pH 7.0 is 280 M^{-1} s^{-1}. The ratio of these two experimental values, 3, is within a factor of 2–3.3 of the calculated ratios. This agreement is consistent with similar types of interactions in the cytochrome b_5–Mb and cytochrome b_5–Hr electron-transfer complexes.

Specific intermolecular interactions of the type discussed above may also facilitate reduction of *P. gouldii* cytochrome b_5 by *P. gouldii* NADH–cytochrome b_5 reductase, while simultaneously *inhibiting* reduction of *P. gouldii* metHr by the same reductase (Utecht & Kurtz, 1987). In this fashion "short-circuiting" of electron flow would be prevented.

ACKNOWLEDGMENTS

We thank David Stewart for assistance with the computer graphics and Dr. Ronald Stenkamp for helpful discussions. We thank Dr. Grant Mauk for providing a computer program that calculates values of k_{11}^{corr}.

SUPPLEMENTARY MATERIAL AVAILABLE

Figures depicting linear dependences of observed first-order rate constants on reagent concentrations and tables listing all second-order rate constants (12 pages). Ordering information is given on any current masthead page.

REFERENCES

Adzamli, J. K., Henderson, R. A., Sinclair-Day, J. D., & Sykes, A. G. (1984) *Inorg. Chem. 23*, 3069–3073.

Armstrong, G. D., Ramasami, T., & Sykes, A. G. (1985) *Inorg. Chem. 24*, 3230–3234.

Bradić, Z., & Wilkins, R. G. (1983) *Biochemistry 22*, 5396–5401.

Bradić, Z., Harrington, P. C., & Wilkins, R. G. (1979) *Biochemistry 18*, 889–893.

Bradić, Z., Harrington, P. C., Wilkins, R. G., & Yoneda, G. (1980) *Biochemistry 19*, 4149–4155.

Bradić, Z., Tsukahara, K., Wilkins, P. C., & Wilkins, R. G. (1986) in *Frontiers of Bioinorganic Chemistry* (Xavier, A. V., Ed.) pp 336–344, VCH, Weinheim, FRG.

Brunori, M., Saggese, U., Rotilio, G. C., Antonini, E., & Wyman, J. (1971) *Biochemistry 10*, 1604–1609.

Dawson, J. W., Gray, H. B., Hoenig, H. E., Rossman, G. R., Schredder, J. M., & Wang, R. H. (1972a) *Biochemistry 11*, 461–465.

Dawson, J. W., Gray, H. B., Holwerda, R. A., & Westhead, E. W. (1972b) *Proc. Natl. Acad. Sci. U.S.A. 69*, 30–33.

Freier, S. M., Duff, L. L., Van Duyne, R. P., & Klotz, I. M. (1979) *Biochemistry 18*, 5372–5377.

Garbett, K., Darnall, D. W., Klotz, I. M., & Williams, R. J. P. (1969) *Arch. Biochem. Biophys. 135*, 419–434.

Harrington, P. C., & Wilkins, R. G. (1983) *J. Inorg. Biochem. 19*, 339–344.

Irwin, M. J., Duff, L. L., Shriver, D. F., & Klotz, I. M. (1983) *Arch. Biochem. Biophys. 224*, 473–478.

Keresztes-Nagy, S., & Klotz, I. M. (1965) *Biochemistry 4*, 919–931.

Klotz, I. M., & Kurtz, D. M., Jr. (1984) *Acc. Chem. Res. 17*, 16–22.

Klotz, I. M., Klotz, T. A., & Fiess, H. A. (1957) *Arch. Biochem. Biophys. 68*, 284–289.

Kurtz, D. M., Jr. (1986) in *Invertebrate Oxygen Carriers* (Linzen, B., Ed.) pp 9–21, Springer-Verlag, New York.

Livingston, D. J., McLachlan, S. J., La Mar, G. N., & Brown, W. D. (1985) *J. Biol. Chem. 260*, 15699–15707.

Lukat, G. S., & Kurtz, D. M., Jr. (1985) *Biochemistry 24*, 3464–3472.

Lukat, G. S., Kurtz, D. M., Jr., Shiemke, A. K., Loehr, T. M., & Sanders-Loehr, J. (1984) *Biochemistry 23*, 6416–6422.

Maroney, M. J., Kurtz, D. M., Jr., Nocek, J. M., Pearce, L. L., & Que, L., Jr. (1986) *J. Am. Chem. Soc. 108*, 6871–6879.

Massey, V., & Hemmerich, P. (1977) *J. Biol. Chem. 252*, 5612–5614.

Mauk, A. G., & Gray, H. B. (1979) *Biochem. Biophys. Res. Commun. 86*, 206–210.

Mauk, M. R., Mauk, A. G., Weber, P. C., & Matthew, J. B. (1986) *Biochemistry 25*, 7085–7091.

McLachlan, S. J., La Mar, G. N., & Sletten, E. (1986) *J. Am. Chem. Soc. 108*, 1285–1291.

McLendon, G., & Miller, J. R. (1985) *J. Am. Chem. Soc. 107*, 7811–7816.

Muhoberac, B. B., Wharton, D. C., Babcock, L. M., Harrington, P. C., & Wilkins, R. G. (1980) *Biochim. Biophys. Acta 626*, 337–345.

Nocek, J. M., Kurtz, D. M., Jr., Pickering, R. A., & Doyle, M. P. (1984) *J. Biol. Chem. 259*, 12334–12338.

Pearce, L. L., Kurtz, D. M., Jr., Xia, Y.-M., & Debrunner, P. G. (1987) *J. Am. Chem. Soc. 109*, 7286–7293.

Peterson-Kennedy, S. E., McGourty, J. L., Kalwait, J. A., & Hoffman, B. H. (1986) *J. Am. Chem. Soc. 108*, 1739–1746.

Poulos, T., & Mauk, A. G. (1983) *J. Biol. Chem. 258*, 7369–7373.

Reem, R. C., & Solomon, E. I. (1987) *J. Am. Chem. Soc. 109*, 1216–1226.

Reid, L. S., & Mauk, A. G. (1982) *J. Am. Chem. Soc. 104*, 841–845.

Reid, L. S., Mauk, M. R., & Mauk, A. G. (1984) *J. Am. Chem. Soc. 106*, 2182–2185.

Runnegar, B. (1984) *J. Mol. Evol. 21*, 33–41.

Samuels, G., & Espenson, J. H. (1979) *Inorg. Chem. 18*, 2587–2592.

Sanders-Loehr, J., & Loehr, T. M. (1981) *Adv. Inorg. Biochem. 1*, 235–252.

Scott, R. A., Mauk, A. G., & Gray, H. B. (1985) *J. Chem. Educ. 62*, 932–937.

Segal, M. G., & Sykes, A. G. (1978) *J. Am. Chem. Soc. 100*, 4585–4588.
Shiemke, A. K., Loehr, T. M., & Sanders-Loehr, J. (1986) *J. Am. Chem. Soc. 108*, 2437–2443.
Sieker, L. C., Stenkamp, R. E., & Jensen, L. H. (1982) in *The Biological Chemistry of Iron* (Dunford, H. B., Dolphin, D., Raymond, K. N., & Sieker, L. C., Eds.) pp 161–175, Reidel, New York.
Slaughter, S. R., Williams, C. H., Jr., & Hultquist, D. E. (1982) *Biochim. Biophys. Acta 705*, 228–237.
Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1983) *J. Inorg. Biochem. 19*, 247–253.
Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1984) *J. Am. Chem. Soc. 106*, 618–622.
Stenkamp, R. E., Sieker, L. C., Jensen, L. H., McCallum, J. D., & Sanders-Loehr, J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 713–716.
Utecht, R. E., & Kurtz, D. M., Jr. (1985) *Inorg. Chem. 24*, 4458–4459.
Utecht, R. E., & Kurtz, D. M., Jr. (1987) *Biochim. Biophys. Acta* (in press).
Wherland, S., & Gray, H. B. (1977) in *Biological Aspects of Inorganic Chemistry* (Addison, A. W., Cullen, W., James, B. R., & Dolphin, D., Eds.) pp 289–368.
Wherland, S., Holwerda, R. A., Rosenberg, R. C., & Gray, H. B. (1975) *J. Am. Chem. Soc. 97*, 5260–5262.
Wilkins, R. G., & Harrington, P. C. (1983) *Adv. Inorg. Biochem. 5*, 51–85.

Structural Elucidation of the *Brucella melitensis* M Antigen by High-Resolution NMR at 500 MHz†

David R. Bundle,*,‡ John W. Cherwonogrodzky,§ and Malcolm B. Perry‡
Division of Biological Science, The National Research Council of Canada, Ottawa, Canada K1A 0R6, and Agriculture Canada, Animal Disease Research Institute, Nepean, Ontario, Canada K2H 8P9

Received April 29, 1987; Revised Manuscript Received July 7, 1987

ABSTRACT: The *Brucella* M antigen from the species type strain *Brucella melitensis* 16M has been identified as a component of the cell wall lipopolysaccharide (LPS). O polysaccharide liberated from this LPS by mild acid hydrolysis exhibited M activity in serological tests and was shown to be a homopolymer of 4-formamido-4,6-dideoxy-α-D-mannopyranosyl residues arranged in an oligosaccharide repeating unit as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the native lipopolysaccharide. Structural analysis of the O polysaccharide by NMR methods was difficult due to apparent microheterogeneity of the repeating unit, which was in fact caused by the presence of rotational isomers of the *N*-formyl moiety. This problem was resolved by chemical modification of the polysaccharide to its amino and *N*-acetyl derivatives, the 500-MHz ^{1}H and 125-MHz ^{13}C NMR spectra of which could be analyzed in terms of a unique structure through application of pH-dependent β-shifts and two-dimensional techniques that included COSY, relayed COSY, and NOESY experiments together with heteronuclear C/H shift correlation spectroscopy. On the basis of these experiments and supported by methylation and periodate oxidation data, the structure of the M polysaccharide was determined as a linear polymer of unbranched pentasaccharide repeating units consisting of four 1,2-linked and one 1,3-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues. The marked structural similarity of the M antigen and the A antigen, which is known to be a 1,2-linked homopolysaccharide of 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units, accounts for cross-serological reactions of the two and the long-standing confusion surrounding the nature of their antigenic determinants. Structural and serological considerations in conjuction with the sodium dodecyl sulfate banding pattern of *Brucella* A LPS suggest that its biosynthesis differs appreciably from that of the M antigen, which appears to be synthesized by regulated assembly of preformed oligosaccharide repeating units. Temperate, lysogenic phage may be responsible for such biosynthetic and structural variations.

Serological detection plays a major role in the routine diagnosis of brucellosis, because it is not always possible to isolate the causative organism (Alton et al., 1975). Although antibodies directed against cell-surface polysaccharides of smooth *Brucella* form the basis of these serological tests, until recently little was known about their chemistry. Two cell wall antigens, A and M, have been recognized in smooth strains of *Brucella* (Wilson & Miles, 1932), and originally it was proposed that both antigens occurred as components of a single AP substance (aminopolyhydroxy compound) that also contained formic acid (Miles & Pirie, 1939; Ellwood et al., 1967). Although there has been ample evidence to suggest that the A and M antigens of *Brucella* correspond to the somatic antigens of the bacterial lipopolysaccharide, LPS[1] (Diaz et al., 1968; Moreno et al.,

† Issued as NRCC Publication No. 28345. Support from NATO Research Grant RG 85/0021 to D.R.B. was instrumental in facilitating access to computing facilities at the Technical University of Denmark, Lyngby, and at the same time permitted the use there of a Bruker AM-500 spectrometer funded by the Danish Research Council, which was one of two such spectrometers used for this study.
* Author to whom correspondence should be addressed.
‡ The National Research Council of Canada.
§ Animal Disease Research Institute.

[1] Abbreviations: NAc-PS, N-deformylated and N-acetylated M polysaccharide; COSY, ^{1}H–^{1}H shift-correlated spectroscopy; GC–MS, gas–liquid chromatography–mass spectroscopy; GLC, gas–liquid chromatography; LPS, lipopolysaccharide; *s*LPS, smooth LPS; NH_2-PS, N-deformylated M polysaccharide; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; Rha4NFo, 4,6-dideoxy-4-formamido-D-rhamnopyranose; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/87/0426-8717$01.50/0 Published 1987 by the American Chemical Society