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Reactivity patterns for redox reactions of monomer forms of myoglobin, hemocyanin and hemerythrin

Bao-Jian Zhang, Colin R. Andrew, N.P. Tomkinson and A. Geoffrey Sykes

Department of Chemistry, The University, Newcastle upon Tyne (UK)

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Electron-transfer reactions of myoglobin, hemocyanin and hemerythrin with the inorganic complexes $[Fe(CN)_6]^{3-}$ (oxidant) and $[Co(sep)]^{2+}$ (reductant) are considered. Rate constants k_{Fe} (25°C) have been determined for the $[Fe(CN)_6]^{3-}$ (410 mV) oxidation of horse deoxyMb, $I = 0.100$ M (NaCl). From the decrease in k_{Fe} over the range pH 5.5 to 9.0 a pK_a of < 6.2 is obtained, most likely due to the involvement of the heme propionate(s). At the higher pH values the rate constant is $1.2 \cdot 10^6$ M^{-1} s^{-1}. Rate constants k_{Co} (25°C) for the $[Co(sep)]^{2+}$ (−260 mV) reduction of metMb are also pH-dependent, $pK_a = 8.82$, corresponding to acid dissociation of the H_2O axially coordinated to the Fe(III). The rate constant for the aqua-met form is $2.8 \cdot 10^3$ M^{-1} s^{-1} at pH values < 7.0. In contrast, no reaction is observed for the deoxy and met forms of *P. interruptus* hemocyanin monomer subunit **a** with the same two complexes ($k < 10^2$ M^{-1} s^{-1}). Comparisons are made with rate constants for hemerythrin, also as the monomer, which have been determined previously. Rate constants for the reactions of deoxy forms with the neutral small molecules, here O_2 and H_2O_2, are also considered. Whereas the reactions of $[Fe(CN)_6]^{3-}$ and $[Co(sep)]^{2+}$ are at the protein surface, those of O_2 and H_2O_2 are at the active site. Hemocyanin with the more buried (~ 20 Å) active site compared with myoglobin (3.8 Å) and hemerythrin (6.3 Å), does not readily undergo electron transfer with reagents at the surface. However, with the small molecules O_2 and H_2O_2 penetration of the surrounding peptide occurs, with reaction at the active site. Rate constants for the three proteins are now of similar magnitude, and in the range $(2.3–7.8) \cdot 10^7$ M^{-1} s^{-1} for O_2, and 10.9 to 3600 M^{-1} s^{-1} for H_2O_2.

Introduction

This paper reports studies on the redox reactivity of met and deoxy forms of horse myoglobin (Mb) and *P. interruptus* hemocyanin (Hc) with $[Fe(CN)_6]^{3-}$ (as oxidant) and $[Co(sep)]^{2+}$ (as reductant) [1]. With information from other studies comparisons of the redox reactivities of the three O_2-binding proteins (as monomers) myoglobin (M_r 16000; 1 Fe and 153 amino acids) [2], hemocyanin (M_r 78000; 2 Cu's and 657 amino acids) [3], and hemerythrin (Hr) (M_r 13800; 2 Fe's and 118 amino acids) [4], are made. Attention is drawn to two quite different catagories of redox reaction, to electron-transfer at the surface, and small molecule reactions (here O_2 and H_2O_2) at the active site. Hemerythrin is the smallest of the proteins. From amino-acid compositions both Mb (horse) and hemerythrin (*T. zostericola*) are estimated to be neutral in charge at pH ≈ 7, with Mb (11 histidines) developing a substantial positive charge at pH ≈ 5. On the other hand, *P. interruptus* hemocyanin, (here subunit a of a, b and c was studied), has a substantial negative charge at pH ≈ 7. X-ray crystal structures have been determined for deoxyMb (sperm whale) [5], metMb (horse and sperm whale) [6,7], deoxyHc from *P. interruptus* [8], deoxyHr (*T. dyscritum*, octamer) [9], azido-metHr (monomer *T. zostericola* and octamer *T. dyscritum*) [10,11], and metHr (*T. dyscritum*) [11]. The monomer proteins have single polypeptide chains which in all three cases have substantial helical structures (70–75% in the case of Hr) [12]. This confers a certain flexibility on the protein, which is believed to help small molecules access and bind to the metal at the active site. Of the three, myoglobin is the only porphyrin-containing protein. The binuclear Cu site in hemocyanin is coordinated by six, and the binuclear Fe site of hemerythrin by five, histidines. In the case of Mb and Hr, auto-oxidation occurs to the met states, Fe(III) and $Fe(III)_2$ respectively. Such effects are kept in check by reductase systems involving NADH and cytochrome b_5 [13–16].

Correspondence to: A.G. Sykes, Department of Chemistry, The University, Newcastle upon Tyne NE1 7RU, UK.

Hemocyanin is, however, extracellular, and does not appear to undergo auto-oxidation.

OxyMb, which equilibrates as in Eqn. 1:

$$MbO_2 \rightleftharpoons deoxyMb + O_2 \quad (1)$$

has been used in this study as an in-situ source of deoxyMb. The equilibrium constant K for (1) is $5.3 \cdot 10^5$ M at 25°C. This value was obtained from $7.8 \cdot 10^5$ M^{-1} at 20°C using $\Delta H = -13.7$ kcal mol^{-1} for horse Mb [17]. All solutions were in air at 25°C, $I = 0.100$ M (NaCl), when $[O_2] = 2.63 \cdot 10^{-4}$ M. Commercially available metMb, purified by anion-exchange chromatography, was used as the source of oxidised protein.

For the studies on hemocyanin in situ deoxyHc and isolated deoxyHc were both used. MetHc was obtained by addition of H_2O_2 to oxyHc, when H_2O_2 oxidation of the deoxyHc component occurs.

$$deoxyHc + H_2O_2 \rightarrow metHc + H_2O \quad (2)$$

EXAFS measurements on metHc from three sources (*Cancer irroratus*, *Limulus polyphemus*, and *Busycon canaliculatum*) give Cu-Cu distances in the range 3.39–3.45 Å, consistent with a μ-hydroxo rather than di-μ-hydroxo structure for metHc [18].

Experimental procedures

Myoglobin

Oxymyoglobin was obtained from lyophilised horse skeletal muscle metmyoglobin (Sigma). The various purification procedures were similar to those described by Shikama and colleagues [19,20]. The protein (0.5 g) was dissolved in a minimum amount of air-free 5 mM Tris-HCl (pH 8.4) (to minimise auto-oxidation), and reduced with sodium dithionite (10 mg; BDH, GPR). Excess dithionite was removed by dialysis against 1000 ml of the same buffer in air, when oxymyoglobin was formed. The protein was loaded onto a Whatman DEAE 23 column (50 × 3 cm) equilibrated in 5 mM Tris-HCl (pH 8.4). The first major oxyMb fraction eluted as a bright red band at 50 mM Tris-HCl, and other bands eluted at higher ionic strength. The first fraction was diluted (×3), loaded onto a DEAE 23 column (30 × 3 cm) equilibrated in 1 mM Tris-HCl, and eluted with 50 mM Tris-HCl. A brown-red metMb fraction eluted ahead of the oxyMb. Protein having an absorbance (A) peak ratio $A_{581}/A_{543} > 1.04$ was used in kinetic studies. Protein concentrations were determined at 418 nm, $\epsilon = 1.33 \cdot 10^5$ M^{-1} cm^{-1} [20].

To obtain metMb no dithionite was added in the above procedure, and the same column purifications were carried out. Protein concentrations were determined at 409 nm, $\epsilon = 1.88 \cdot 10^5$ M^{-1} cm^{-1} [21].

Hemocyanin

Hemolymph from the spiny lobster (*Panulirus interruptus*) supplied by the Pacific Bio-Marine Laboratories (Venice, CA, USA) without added anti-coagulant, was purified by a procedure generously provided by Dr. H. Bak at the University of Groningen, Netherlands. Details of the procedure and separation of monomer subunit a of oxyHc are as described in Ref. 22.

To convert to deoxyHc, a solution of oxyHc (typically 2 ml of $2 \cdot 10^{-4}$ M) was dialysed against 500 ml of 0.05 M Tris-HCl and 0.005 M Na_2H_2edta (edta^{4-}, ethylenediaminetetraacetate) at pH 8.7, which had been made air-free by bubbling N_2 gas through the solution for 6 h at 4°C. Sodium dithionite (Fluka) 0.5 g/l was then added, and the solution dialysed against buffer for 8 h at 4°C. Excess reductant was removed by dialysis against a 300-fold excess (v/v) of air-free buffer, with three changes, in a glove box. Protein concentrations were determined by absorbance measurements at 280 nm, $\epsilon = 1.04 \cdot 10^5$ M^{-1} cm^{-1} per binuclear Cu centre [23], or exposure to air and determination as oxyHc.

To obtain metHc a 50-fold excess of H_2O_2 (BDH, Analar) was added to a solution of oxyHc in 0.05 M Tris-HCl containing 0.005 M Na_2H_2edta at pH 8.7 (20 h, approx. 20°C) and excess oxidant removed by dialysis against a 300-fold excess (v/v) of the required buffer, which was changed three times. Protein concentrations were determined by absorption measurements at 337 nm, assuming $\epsilon = 3700$ M^{-1} cm^{-1} per Cu^{2+}. This value was obtained by quantitative conversion of oxyHc as in (2), where oxyHc has $\epsilon = 2.0 \cdot 10^4$ M^{-1} cm^{-1} at 337 nm [24]. MetHc denatured upon freeze/thawing as judged by changes in the UV-VIS spectrum, and solutions were therefore freshly prepared for each experiment and used within 2 days.

Complexes

Potassium ferricyanide, $K_3[Fe(CN)_6]$ (BDH, AnalaR), was used without further purification. Concentrations were determined spectrophotometrically at the 420 nm peak ($\epsilon = 1010$ M^{-1} cm^{-1}). The compound $[Co(sep)]Cl_3 \cdot H_2O$, where 'sep' denotes the sepulchrate cage ligand 1,3,6,8,10,13,16,19-octaazobicyclo-[6.6.6]eicosane, was prepared as described in Ref. 25 and converted to the sulphate salt prior to electrochemical reduction. To do this $(Co(sep)]Cl_3 \cdot H_2O$ (1 g) was dissolved in conc. H_2SO_4 (2.5 ml). After 1–2h the solution was poured into methanol (50 ml), the precipitate filtered off, and the compound recrystallised from 0.010 M H_2SO_4 (10–15 ml, 75°C). A mixed sulphate/hydrogen sulphate salt, formula $[Co(sep)]SO_4 \cdot HSO_4 \cdot 2H_2O$ was obtained. Electrochemical reduction was carried out at a potential of −0.8 V (vs SCE). Solutions of $[Co(sep)]^{3+}$ (10 mM) in 20 mM Tris-H_2SO_4 required about 5 h for reduction.

Reduction potentials

These have been reported (vs. nhe) for the Mb Fe(III)/Fe(II) couple as low as 5 mV (see below) [26], and for the Hr change $Fe(III)_2/Fe(III)Fe(II)$ (110 mV) [27]. There are no values as yet for hemocyanin. Known values for binuclear Cu proteins are tyrosinase (360 mV) [28]. *Rhus vernicifera* (tree) laccase (434 mV), and *Polyporus versicolour* (fungal) laccase (782 mV) [29]. The complexes $[Fe(CN)_6]^{3-}$ and $(Co(sep)]^{2+}$ were selected as probes for electron-transfer reactivity because of their charge and E^0 values, $[Fe(CN)_6]^{3-/4-}$ (410 mV), and $[Co(sep)]^{3+/2+}$ (−260 mV) [25].

Buffers

The following buffers, all from the Sigma, were used: 2-(*N*-morpholino)ethanesulphonic acid (Mes), pH 5.5–7.0; 3-(*N*-morpholino)propanesulphonic acid (Mops), pH 6.2–7.6; *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulphonic acid (Hepes) pH 6.7–8.2; tris(hydroxymethyl)aminomethane (Tris), pH 7.20–9.00; and 2-(*N*-cyclohexylamino)ethanesulphonic acid (Ches), pH 8.6–10.0.

Procedure

Solutions of oxyMb were dialysed into 2 mM Tris-HCl buffer at pH 8.4, $I = 0.100$ M (NaCl). The pH-jump procedure was used for kinetic experiments at different pH values, with the inorganic reagent in a larger (dominant) concentration of buffer (50 mM), so that the pH of the latter applied.

Solutions of Hc were dialysed against 50 mM Tris-HCl containing 5 mM Na_2H_2edta at pH 8.7, $I = 0.100$ M (NaCl). Under these conditions the protein is present as monomer.

Rigorous air-free conditions were employed in all studies involving reduction of met protein. All samples of oxy protein were in air-saturated solutions. The concentration of O_2 in such solutions is $2.63 \cdot 10^{-4}$ M, as determined using a Beckman 0260 O_2-analyzer complete with O_2 sensor cathode.

All the Mb kinetic studies were carried out using a Dionex D-110 stopped-flow spectrophotometer. The reaction of deoxyMb with $[Fe(CN)_6]^{3-}$ was followed by monitoring the decay of oxyMb at 582 nm. The $[Co(sep)]^{2+}$ reduction of metMb was monitored by the absorbance decrease at 502 nm. Inorganic reagents were in at least 10-fold excess of the protein. First-order rate constants, k_{obs} were obtained using fitting procedures from On-Line Instruments Systems (Jefferson, GA, USA). Second-order rate constants were obtained from a linear or non-linear least-squares treatment as appropriate. Experiments with Hc were monitored on a Shimadzu UV-21 01 PC spectrophotometer attached to an RM Nimbus VX/2 PC.

All reactions were at 25.0 ± 0.1°C, and with ionic strength $I = 0.100$ M (NaCl).

TABLE 1

Rate constants (25 °C) for the oxidation of horse deoxyMb ($c \approx 1.0 \cdot 10^{-5}$ M) with $[Fe(CN)_6]^{3-}$, $I = 0.100$ M (NaCl)

pH	$10^3[Fe(CN)_6^{3-}]$ (M)	k_{obs} (s^{-1})	$10^{-3} k_{app}$ ($M^{-1} s^{-1}$)	$10^{-6} k_{Fe}$ ($M^{-1} s^{-1}$)
5.7 (Mes)	4.54	9.0	1.98	2.76
6.0 (Mes)	0.43	0.77		
	0.61	1.05		
	0.87	1.51		
	1.73	2.74		
	4.33	6.6		
	4.54	7.2		
	5.62	8.7	1.52 [b]	2.21 [b]
6.5 (Mes)	4.54	6.2	1.37	1.91
7.0 (Hepes)	1.78	2.01		
	3.48	3.7		
	4.54	4.9		
	6.08	6.4	1.02 [b]	1.42 [b]
7.5 (Hepes)	4.54	4.5	0.99	1.38
8.0 (Hepes)	0.67	0.65		
	1.69	1.65		
	2.36	2.30		
	4.54	4.2		
	5.88	5.7	0.92 [b]	1.28 [b]
8.5 (Tris)	4.54	4.0	0.88	1.23
9.0 (Ches)	0.94	0.83		
	1.88	1.70		
	2.63	2.24		
	4.35	3.8		
	4.54	3.9		
	6.09	5.2	0.84 [b]	1.17 [b]

[a] Using $K = 5.3 \cdot 10^5$ M and $[O_2] = 2.63 \cdot 10^{-4}$ M. [b] Using all data obtained at this pH.

Results

$[Fe(CN)_6]^{3-}$ Oxidation of DeoxyMb

Solutions of oxyMb were used as a source of deoxyMb. Stopped-flow traces indicate a single rate-determining step. First-order equilibration rate constants k_{obs}, with $[Fe(CN)_6]^{3-}$ in large excess, are listed in Table I. The behaviour observed is consistent with the reaction sequence in 3, 4,

$$MbO_2 \rightleftharpoons \text{deoxyMb} + O_2 \tag{3}$$

$$\text{deoxyMb} + [Fe(CN)_6]^{3-} \rightarrow \text{metMb} + [Fe(CN)_6]^{4-} \tag{4}$$

where reaction 3 is rapid. Apparent second-order rate constants, k_{app}, obtained from the linear dependence of k_{obs} on $[Fe(CN)_6]^{3-}$, gave rate constants k_{Fe} from the dependence Eqn. 5,

$$k_{Fe} = K k_{app} [O_2] \tag{5}$$

making due allowance for reaction 3. The pH dependence of k_{Fe} is illustrated in Fig. 1. At pH > 8.2 k_{Fe} is $1.2 \cdot 10^6$ $M^{-1} s^{-1}$. No data could be obtained at pH <

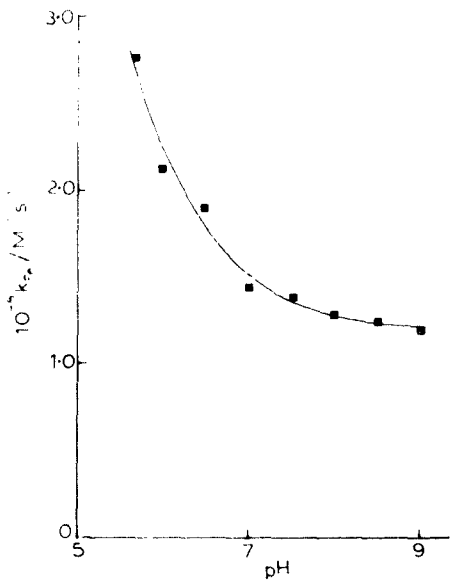

Fig. 1. The variation of second-order rate constants k_{Fe} (25 °C) for the $[Fe(CN)_6]^{3-}$ oxidation of the horse deoxyMb component of oxyMb with pH. I = 0.100 M (NaCl). The trend in rate constants only is illustrated, no mathematical fit has been carried out.

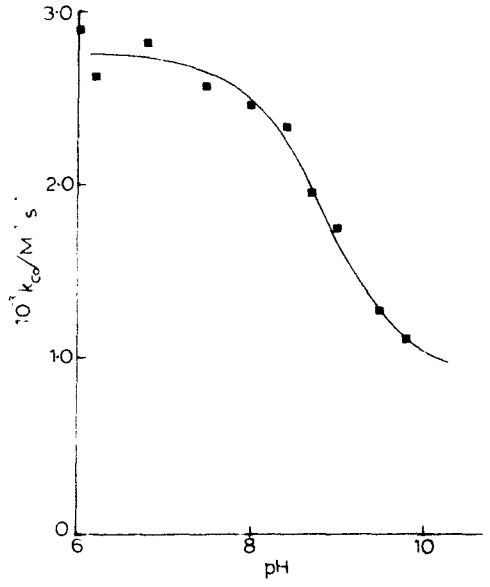

Fig. 2. The variation of second-order rate constants k_{Co} (25°C) for the $[Co(sep)]^{2+}$ reduction of horse metMb with pH. I = 0.100 M (NaCl). The solid line drawn is in accordance with the least-squares fit to Eqn. 9.

5.7 due to protein denaturation. From Fig. 1, assuming a single process to be relevant, the pK_a is < 6.2.

$[Co(sep)]^{2+}$ reduction of MetMb

First-order rate constants k_{obs} for the single-stage, stopped-flow reaction, are listed in Table II. At pH 8.4 a linear dependence on $[Co(sep)]^{2+}$ present in > 10-fold excess was demonstrated. Hence, second-order rate constants k_{Co} were obtained, (Table II). The effect of pH is illustrated in Fig. 2. Previously it has been shown that the axial H_2O of horse metMb has a pK_a of 8.82 at I = 0.10 M (NaCl) [30]. Accordingly a reaction sequence (6)–(8) is proposed,

$$metMb(H_2O) \rightleftharpoons metMb(OH^-) + H^+ \quad (6)$$

$$metMb(H_2O) + [Co(sep)]^{2+} \rightarrow deoxyMb + [Co(sep)]^{3+} \quad (7)$$

$$metMb(OH) + [Co(sep)]^{2+} \rightarrow deoxyMb + [Co(sep)]^{3+} \quad (8)$$

from which (9) is obtained.

$$k_{Co} = \frac{k_1[H^+] + k_2K}{[H^+] + K_a} \quad (9)$$

A non-linear least-squares fit gives $pK_a = 8.82 \pm 0.07$, in excellent agreement with the literature value [30]. The rate constants $k_1 = (2.75 \pm 0.04) \cdot 10^3$ M^{-1} s^{-1} and $k_2 = (0.95 \pm 0.08) \cdot 10^3$ M^{-1} s^{-1} are also obtained.

TABLE II

Rate constants (25° C) for the reduction of horse metMb ($\sim 1.0 \cdot 10^{-5}$ M) with $[Co(sep)]^{2+}$, I = 0.100 M (NaCl)

pH	$10^3[Co(sep)^{2+}]$ (M)	k_{obs} (s^{-1})	$10^{-3}k_{Co}$ ($M^{-1}s^{-1}$)
6.0 (Mes)	1.19	3.4	2.86
6.2 (Mes)	2.12	5.7	2.69
6.8 (Mops)	1.29	3.7	2.87
7.5 (Mops)	1.28	3.3	2.58
8.0 (Tris)	2.11	5.2	2.46
8.4 (Tris)	0.46	1.33	
	0.97	2.12	
	1.56	3.5	
	1.90	4.6	
	2.64	6.2	
	4.17	9.8	2.34
8.7 (Ches)	1.93	3.8	1.97
9.0 (Ches)	1.18	1.97	1.67
9.5 (Ches)	1.88	2.40	1.28
9.8 (Ches)	2.12	2.35	1.11

Reactivity of Hc

Absorbances were monitored in the 300–850 nm range by conventional spectrophotometry. In sharp contrast to the above, no reaction of $[Fe(CN)_6]^{3-}$ ($3 \cdot 10^{-3}$ M) with deoxyHc ($3 \cdot 10^{-5}$ M) was observed at pH 8.7 over 48 h at 25°C. Similarly, no reaction of metHc ($8 \cdot 10^{-5}$ M) with $[Co(sep)]^{2+}$ ($5.5 \cdot 10^{-5}$ M) was observed at pH 8.7 over 4 h. Although the latter experiments were under air-free conditions using rubber septum, N_2 and syringe techniques, some air gained

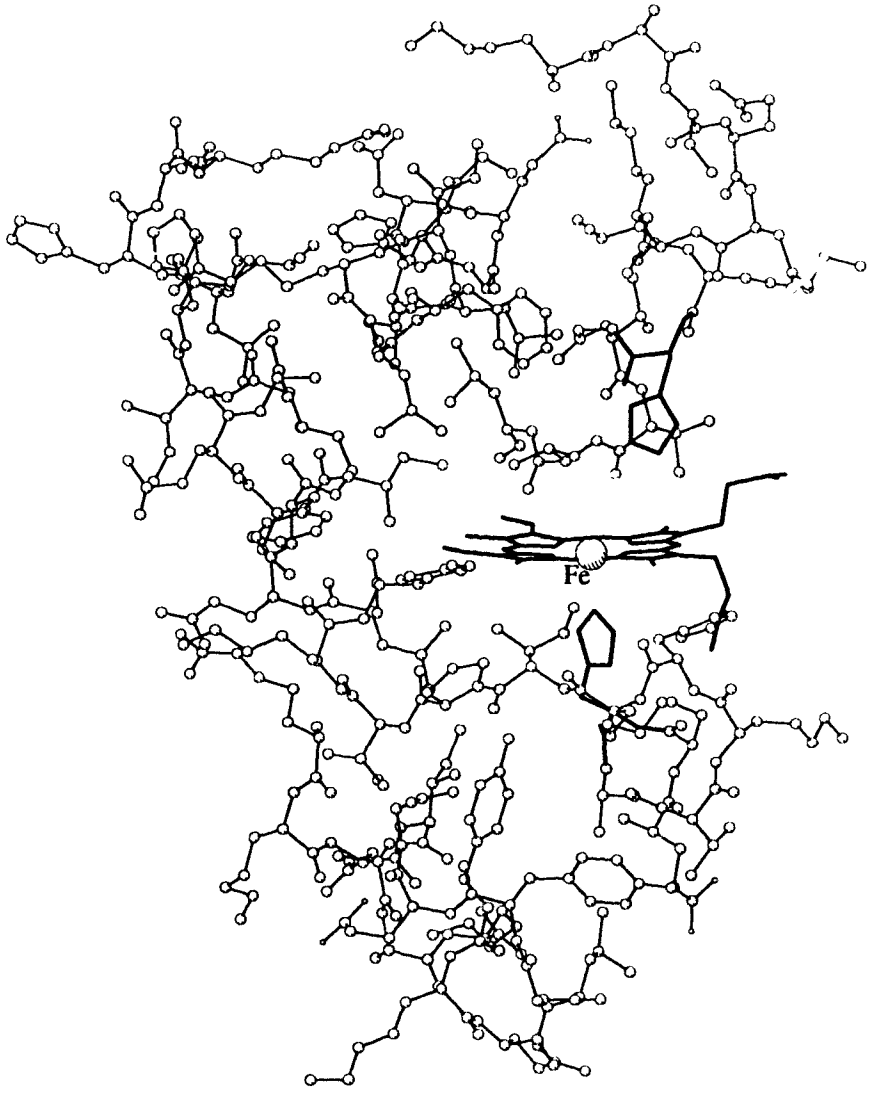

Fig. 3. Structure of metMb (sperm whale) from data in Ref. 6, showing the heme Fe and attached propionates at positions 6 (far) and 7 (near) positions.

access over longer reaction times. From these observations rate constants are $< 10^{-2}$ M^{-1} s^{-1}.

Discussion

The redox inactivity of Hc, in this instance with $[Fe(CN)_6]^{3-}$ and $[Co(sep)]^{2+}$, is a particularly important feature. From the X-ray crystal structure the binuclear Cu active site in *P. interruptus* Hc[8] is buried and about 20 Å from the closest point of the surface. This is much larger than the corresponding distances for Mb (3.8 Å) and Hr (6.3 Å). The reduction potential of Hc is not known because of its redox inertness. Estimates from values for other binuclear Cu proteins suggests a value in the 300–800 mV range [28,29]. It is possible, therefore, that $[Fe(CN)_6]^{3-}$ (410 mV) is not reactive because it does not have a sufficiently high potential. If this is the case then $[Co(sep)]^{2+}$ (−260 mV) should have an enhanced reducing capacity for metHc. That no reaction is observed in either case indicates that the most likely explanation is the buried nature of the active site.

In the case of Mb, effects of pH were studied in order to define the precise form of the protein reactant. Rate constants reported are in good agreement with literature values, which have been reported at a single pH value [31–33]. It is possible that in the $[Fe(CN)_6]^{3-}$ oxidation of deoxyMb, the pH effect observed is due to protonation of one or both of the propionates at positions 6 and 7 on the heme ring. The

Fig. 4. Space-filling model of metMb from data in Ref. 6, showing the exposed heme edge with the propionate groups at positions 6 (to the right) and 7 (to the left).

orientation of these is shown in Fig. 3, and again in the space-fitting model, Fig. 4, which indicates the extent of exposure of the heme edge, and the inaccessibility of the Fe. The exposed heme edge situation is similar to that applying in the case of the cytochromes. The

TABLE III

Comparison of rate constants (M^{-1} s^{-1}, 25°C) at pH ≈ 8.5 for reactions of horse myoglobin (Mb), T. zostericola monomer form of hemerythrin (Hr), and P. interruptus monomer subunit a form of hemocyanin (Hc)

The $[Co(sep)]^{2+}$ entries are for reaction with met protein, the others for the reactions of deoxy forms.

Reactant	k(Mb) [a]	k(Hc) [a]	k(Hr) [b]
$[Fe(CN)_6]^{3-}$	$1.2 \cdot 10^{6}$ [c]	No reaction [c]	$> 3 \cdot 10^{6}$ [d]
$[Co(sep)]^{2+}$	$2.8 \cdot 10^{3}$ [c]	No reaction [c]	$2.2 \cdot 10^{3}$ [e]
O_2	$2.3 \cdot 10^{7}$ [f]	$4.6 \cdot 10^{7}$ [g]	$7.8 \cdot 10^{7}$ [h]
H_2O_2	$3.6 \cdot 10^{3}$ [i]	10.9 [a]	97 [j]

[a] $I = 0.100$ M (NaCl); [b] $I = 0.150$ M (Na_2SO_4); [c] This work; [d] Ref 37; for *T. zostericola* octamer $k = 1.5 \cdot 10^{5}$ M^{-1}, Ref. 38. [e] At pH 6.3 when active site is metaqua form, Ref. 39; [f] Sperm whale Mb, Ref. 40, value not known for horse Mb; [g] Ref. 22; [h] Ref. 41; [i] Sperm whale Mb, Ref. 19, value not known for horse Mb; [j] Ref. 42; for the octamer $k = 5.5$ M^{-1} s^{-1}, Ref. 43.

propionates are strategically placed and believed to be influential. Protonation is seen to enhance rate constants for the reaction with $[Fe(CN)_6]^{3-}$ consistent with an effect of charge. There are, however, a number of histidine residues, and as these protonate, some additional enhancement in rate constants is likely. From Fig. 1 it can be concluded that, for a single dominant acid dissociation process, the pK_a will be < 6.2. Previously determined metMb pK_a values are about 5.3 for the 6-propionate [34], and 5.3 or lower for the 7-propionate [35]. Fig. 5 indicates the closest approach.

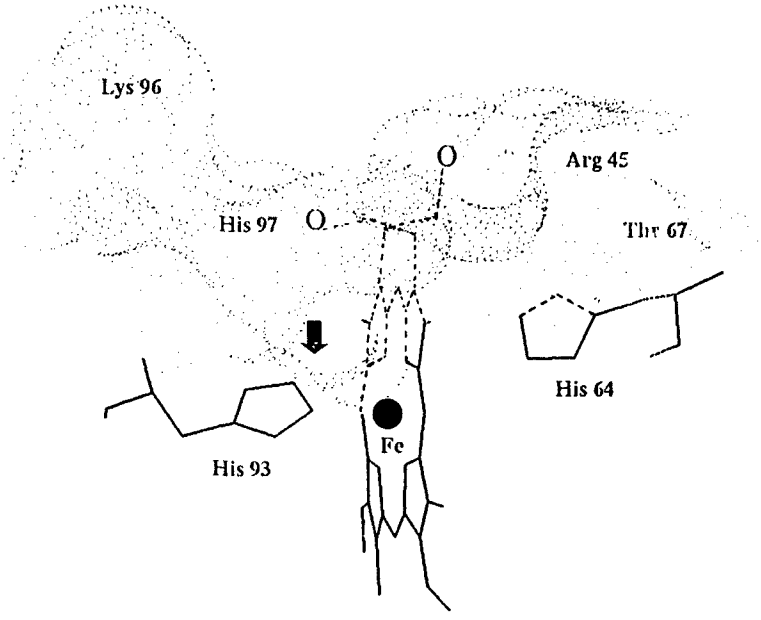

Fig. 5. The proximity of the heme Fe active site of metMb to the surface, from Ref. 6. The arrow indicates the closest point of the surface to the Fe (distance 3.8 Å). The proximal (His-93) and distal (His-64) residues, heme propionates, and surface amino acid residues are indicated.

Interpretation of the effect of pH in the case of the $(Co(sep)]^{2+}$ reduction of metMb is more straightforward. The pK_a corresponds to acid dissociation of the axially coordinated H_2O. This H_2O is absent in the case of 5-coordinate deoxyMb. It is reasonable that the aqua-met form is more reactive because of the greater ease of bond stretching of Fe-H_2O, as compared to Fe-OH. Consistent with these observations, the E^0 of horse metMb has been reported to be about 70 mV at pH 6.0 (the aqua form), and about 30 mV at pH 9.5 (the hydroxo form) [36].

Rate constants for the reactions of $[Fe(CN)_6]^{3-}$ and $[Co(sep)]^{2+}$ with deoxyHr and metHr have been determined previously [39]. Dependencies on $[H^+]$, which are observed over the pH range 6–9, have been discussed. That for the first stage of the reduction of metHr is, for example, like metMb, assigned to the different reactivity of the aqua and hydroxo forms and gives a 40% decrease. The reactivities of the monomer forms of Mb, Hc and Hr, are summarised in Table III [37–43]. The rate constants for Mb and Hr are, in fact, quite similar. From E^0 values the driving force is more favourable for the $[Co(sep)]^{2+}$ reduction of metHr than for metMb, although the active site is not as close to the surface. The precise region(s) of the surface at which the redox partners bind and electron transfer occurs are not known, and will presumably be determined by the location of charged residues, some of which are shown in Figs. 5 and 6 [6,44], as well as favourable routes for electron transfer.

From studies on the interactions of inorganic complexes with proteins there is no evidence, e.g., from NMR [45], for changes in the structure of the protein corresponding to substantial penetration of the protein surface by such complexes. Solvation effects at the protein surface, as well as the complex, will create a barrier to such penetration.

It is quite extraordinary that Hc should be so unreactive with the representative inorganic complexes here tested, and yet be so reactive with O_2 and H_2O_2. Indeed the rate constant for Hc with O_2 [22], is very similar to those for Mb and Hr [40,41], Table III. There is a somewhat wider spread of rate constants for reaction with the 4-atom H_2O_2 [42–43]. In the case of Hc and Hr, the O_2 molecule is bound as a peroxo ligand, with corresponding $Cu(I)_2 \rightarrow Cu(II)_2$ and $Fe(II)_2 \rightarrow Fe(III)_2$ changes in oxidation state. The active site of oxyMb can be regarded as approximating to Fe(III)-O_2 , but alternative views have been presented [46]. The reaction of deoxyMb with H_2O_2 is believed to proceed via an Fe(IV) intermediate [19]. In the reaction of O_2 with deoxyMb one view is that O_2 enters the protein matrix and accesses the active site by different routes in the vicinity of Val68(E11) and the distal histidine His64(E7) [47].

To summarise, both the O_2 and H_2O_2 small molecule reactants can be regarded as inner-sphere oxidants, whereas the reactions with $[Fe(CN)_6]^{3-}$ and $[Co(sep)]^{2+}$ are of the outer-sphere type. This is a clear-cut example, therefore, in which these terms can usefully be applied to metalloprotein reactions. Hemocyanin is most reactive with small molecules which can access the active site.

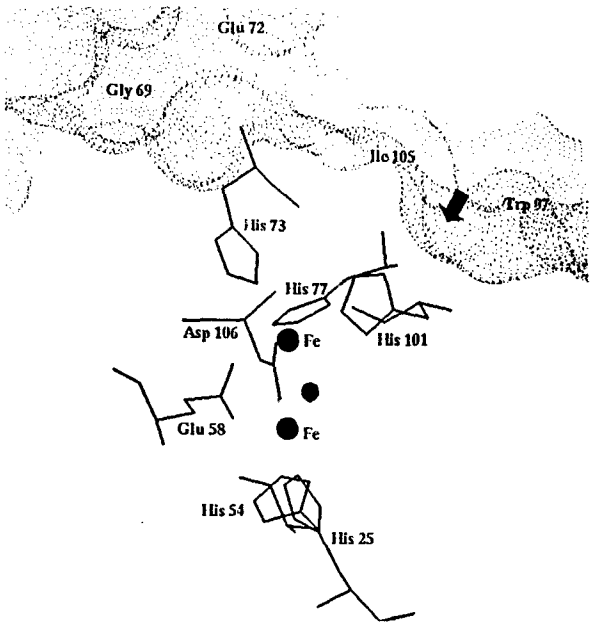

Fig. 6. The proximity of the binuclear Fe active site of metHr to the surface, from the structure coordinates in Ref. 44. The arrow indicates the closest point of the surface to the nearest Fe (distance 6.3 Å). The μ-oxo group (unlabelled solid circle), active site ligands, and surface amino acid residues are indicated.

Hemocyanin on its own provides an interesting contrast. While it is able to carry out its O_2-carrying function as efficiently as myoglobin and hemerythrin, it does not readily undergo auto-oxidation. Both myoglobin and hemerythrin are intracellular and have access to reductase systems to retrieve the met form, whereas hemocyanin exists as an extracellular protein with no known reductase.

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