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Reduction of Methemerythrin by Deoxymyoglobin: a Protein–Protein Redox Reaction Not Involving Electron-Transfer Proteins†

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ABSTRACT: The stoichiometry and kinetics of reaction of methemerythrin with the deoxy forms of myoglobin and hemoglobin have been examined at $I = 0.2$ M and 25 °C. One mole of methemerythrin (on the basis of the monomer unit containing two irons) reacts with 2 mol of deoxymyoglobin and with 0.5 mol of deoxyhemoglobin. All reactions are second order. Rate constants for reaction with deoxymyoglobin are 0.25 M^{-1} s^{-1} (*Phascolopsis gouldii*) and 5.6 M^{-1} s^{-1} (*Themiste pyroides*) at pH 6.3. There is little effect of raising the ionic strength to 1.35 M and only a small decrease in rate when the pH is adjusted to 8.2. The rate constant for reaction of deoxyhemoglobin with *P. gouldii* methemerythrin is ~0.1 M^{-1} s^{-1} at pH 6.3. Metmyohemerythrin from *T. pyroides* reacts slightly slower than the octamer form ($k = 2.0$ M^{-1} s^{-1} at pH 6.3 and 7.0). Oxymyoglobin is converted to metmyoglobin by methemerythrin. The electron-transfer path is discussed and a self-exchange rate constant for hemerythrin assessed as 10^{-3} M^{-1} s^{-1} on the basis of Marcus's theory.

The kinetics of redox reactions involving metalloproteins are being increasingly studied (for recent reviews, see Bennett, 1973; Moore & Williams, 1976; Sutin, 1977; Wherland & Gray, 1977). Most of the work so far involves inorganic reactants, and there are few reported investigations of electron transfer between metalloproteins (Chien et al., 1978; Wherland & Pecht, 1978, and cited references). In even these at least one of the partners appears to be a protein for which electron transfer is an important biological function.

We have been studying a number of redox reactions of hemerythrin and decided to include its reactivity toward myoglobin. Both are well-characterized metalloproteins, whose structures, particularly that of myoglobin, are well understood (Kendrew et al., 1960; Phillips, 1978; Kurtz et al., 1978). Both exist in well-defined iron(II) and iron(III) states and hemerythrin has the added interest of being available both as a monomer and as an octamer, containing two linked irons in each monomeric unit. A noncomplementary redox reaction between two- and one-electron redox reagents is therefore involved. Both proteins in their reduced state function as O_2-storage or O_2-carrying proteins. However, in the oxy form, both autoxidize to the iron(III) species (Brown & Mebine, 1969; Bradić et al., 1977) and redox processes are therefore important. The only reported redox kinetics involving myoglobin and oxymyoglobin with another metalloprotein is that with ferricytochrome *c* (Wu et al., 1972; Ataullakhanov et al., 1976; Atanasov et al., 1977). In this paper we examine the stoichiometry and kinetics of reaction of horse heart and sperm whale deoxymyoglobin with octameric methemerythrin from the coelomic fluid of *Phascolopsis gouldii* and *Themiste pyroides* in the pH 6.3–8.2 range. Some data were also obtained for monomeric metmyohemerythrin from the retractor muscle of *T. pyroides* and for replacing myoglobin by hemoglobin.

Experimental Section

Materials. The marine worms *P. gouldii* and *T. pyroides* were obtained, respectively, from Marine Biological Laboratory, Woods Hole, MA, and Pacific Biomarine Supply, Venice, CA. Oxyhemerythrin was obtained from the coelomic fluid of these worms by the methods of Klotz et al. (1957) and Klippenstein et al. (1972). Metmyohemerythrin was obtained directly from the retractor muscles of *T. pyroides* by a modification of the procedure of Klippenstein et al. (1972), omitting the addition of azide. Methemerythrin was prepared from oxyhemerythrin by adding 2–3 molar excess of $Fe(CN)_6^{3-}$ or by dialyzing against 1 mM $Fe(CN)_6^{3-}$. The product was then dialyzed several times against the appropriate buffer system and absence of iron(II)- or iron(III)–cyano complexes in the final dialysate checked by absorption measurement at 320 or 420 nm. The spectral properties of the various forms agreed with literature descriptions (Klotz, 1971; Klippenstein et al., 1972; Dunn et al., 1977). Sperm whale myoglobin (Sigma) was reduced to the deoxy form by dialyzing overnight at 4 °C against deaerated buffer containing a slight excess of dithionite. The deoxy protein was then dialyzed against buffer to remove excess of dithionite. In one run, oxymyoglobin prepared from the muscle of sperm whale was purified by the method of Keyes et al. (1971) and was then converted into

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deoxymyoglobin by flowing nitrogen slowly over a thin layer of concentrated protein solution. In a number of runs the commercial (met) product (~1 mM) was converted into deoxy in situ by the addition of a slight excess (~10%) of dithionite, then diluted into deaerated buffer, and reacted with methemerythrin. All procedures gave similar results. Horse heart myoglobin (Sigma) and human hemoglobin (Worthington) were converted into deoxy forms by dialyzing against dithionite and several changes of buffers. The met forms were required only to test their unreactivity with deoxyhemerythrin. They were prepared by dialysis of commercial products against a slight excess of $Fe(CN)_6^{3-}$ (on the basis only of the oxy form present) and then several times against buffer.

Measurements. The space above all solutions was extensively flushed with N_2 for some time before spectral measurements were made using a Beckman 24 recording spectrophotometer. In the stoichiometry determinations, 0.12–1.3 mM (on basis of molecular weight of monomer) methemerythrin and 0.07–0.70 mM myoglobin or hemoglobin (on basis of monomer) were mixed in varying ratios, and the absorbances at 505, 560, and 635 nm were measured, before and after reaction. At the completion of the kinetics reactions ($Hr^+ > Mb$),[1] O_2 was admitted and the increase in absorbance at 500 nm (due to the formation of oxyhemerythrin) was measured, thus allowing determination of deoxyhemerythrin produced in the original reaction. In other experiments, azide ion (1.0 mM) was added to the reaction product. The rapid and slower absorbance increases at 475 nm, due to formation of azide adducts of metmyoglobin and methemerythrin, respectively, were measured, and from the known absorbance coefficients of the anion adducts, the separate concentrations of the met forms could be calculated. The kinetic measurements were carried out at 560 nm with rigorous exclusion of O_2 using excess methemerythrin (0.6–3.0 mM) over deoxymyoglobin (0.05–0.15 mM) or deoxyhemoglobin (0.07 mM on basis of monomer unit). Linear semilog plots were obtained, usually up to 3 half-lives. In the study of the reaction of oxymyoglobin (10–50 μM) with methemerythrin (0.4–3.0 mM) in air-saturated solutions, allowance was made for the slower autoxidation of oxy- to metmyoglobin. Experiments at pH 6.3 used 0.03 M Mes and, at pH 7.0 and 8.2, 0.03 M Tris was employed. All runs were at 25 °C and I = 0.2 M, with added Na_2SO_4.

Results

Stoichiometry. Spectral analysis of the product when methemerythrin was reacted with deoxymyoglobin, in a variety of concentration conditions (Table I), shows that all Mb is oxidized when $Hr^+ > Mb$ and that all Hr^+ is reduced to Hr when $Mb > Hr^+$. This confirms that the reaction proceeds according to eq 1; n = 8 or 1. Direct spectral analysis

$$Hr^+(Fe^{III}Fe^{III})_n + 2nMb(Fe^{II}) \rightarrow Hr(Fe^{II}Fe^{II})_n + 2nMb^+(Fe^{III}) \quad (1)$$

monitors predominantly the changes in the concentrations of the myoglobin species, since the hemerythrin forms are weakly absorbing at the analysis wavelengths; e.g., at 560 nm, ϵ_{Hr^+} = 270; ϵ_{Hr} = 130; $\epsilon_{Mb^+} = 3.4 \times 10^3$; $\epsilon_{Mb} = 1.2 \times 10^4$ M^{-1} cm^{-1}. However, the met forms of the proteins could be analyzed in the reaction mixture by adding azide ion to the product. There is a marked difference in the rates of reaction of methemerythrin and metmyoglobin with azide ion (e.g., respective reaction half-lives of 1–2 min and <1 s using 1.0 mM N_3^-, Meloon & Wilkins, 1976; Olivas et al., 1977). This allowed their separate estimation from the biphasic absorption amplitudes and the known absorption coefficients of the metmyoglobin, methemerythrin, and azide adducts. Both *P. gouldii* and *T. pyroides* were examined and results were consistent with eq 1 at both pH 6.3 and 8.2 (Table I). As would be expected, there was no significant spectral change over 18 h at either pH when the products of reaction 1, metmyoglobin and deoxyhemerythrin, were mixed. The analysis of the products of the reaction between *P. gouldii* methemerythrin and deoxyhemoglobin directly at 555 and 630 nm showed quantitative conversion at pH 6.3 according to eq 2.

$$Hr^+(Fe^{III}Fe^{III})_8 + 4Hb(Fe^{II})_4 \rightarrow Hr(Fe^{II}Fe^{II})_8 + 4Hb^+(Fe^{III})_4 \quad (2)$$

Kinetics. Most of the kinetic data were obtained at pH 6.3. Good first-order plots for the disappearance of deoxymyoglobin were obtained when excess methemerythrin (monomeric or octameric) was added to deoxymyoglobin. No rapid initial spectral changes were observed, isosbestic points were maintained (Figure 1) at 667, 597, and 524 nm, which are close to those for the met- or deoxymyoglobin system, and rate constants computed from data at four different wavelengths were within 4% of each other. The pseudo-first-order rate constant, k_{obsd}, varied linearly with methemerythrin concentration (Figure 2). Second-order rate constants at pH 6.3 (and 7.0) for reaction of *P. gouldii* and *T. pyroides* octameric and monomeric are 0.25, 5.6, and 2.0 M^{-1} s^{-1}, respectively. Similar results were obtained when sperm whale was replaced by horse heart myoglobin or when sperm whale oxymyoglobin was carefully purified (a contaminant, called substance K, was removed, Keyes et al., 1971) and the deoxy prepared without recourse to dithionite treatment (Figure 2). There was no

Table I: Spectral Analysis of Products of Reaction of Methemerythrin with Deoxymyoglobin and Deoxyhemoglobin

pH	Hr^+ (mM)[a]	Mb (mM)[a]	absorbance change: theor[b]	absorbance change: exptl
6.3	0.32 P.g[c]	0.72 h.h[d]	−0.65[e,f]	−0.67[f]
6.3	0.54 P.g	0.72 h.h	−0.73[e,f]	−0.67[f]
			+0.08[g]	+0.08
6.3	1.6 P.g	0.72 h.h	+0.26[h]	+0.27
			+0.08[g]	+0.08
6.3	0.25 T.p	0.042 s.w	−0.35[i]	−0.34
6.8	0.18 P.g	0.094 s.w	+0.14[j]	+0.15
			+0.39[k]	+0.42
8.2	0.55 P.g	0.064 s.w	−0.55[e]	−0.51
7.0	0.16 MyoT.p	0.045 s.w	−0.38[e]	−0.37
			+0.046[g]	+0.043
		Hb[a]		
6.3	0.60 P.g	0.067	−0.49[l]	−0.46
6.3	0.13 P.g	0.049	−0.36[l]	−0.37

[a] Initial concentrations of methemerythrin (based on monomer unit), deoxymyoglobin, and deoxyhemoglobin (per heme). [b] On the basis of stoichiometry of reactions 1 and 2. [c] Hemerythrin species: P.g = *P. gouldii*; T.p = *T. pyroides*. [d] Myoglobin species: h.h = horse heart; s.w = sperm whale. [e] At λ = 560 nm, $\Delta\epsilon = 1.03 \times 10^4$ ($Mb - Mb^+$) and 1.4×10^2 ($Hr^+ - Hr$). [f] Solutions diluted tenfold at completion of reaction. [g] Further change at λ = 500 nm on adding O_2 to product, $\Delta\epsilon = 2.2 \times 10^3$ ($HrO_2 - Hr$). [h] At 630 nm, $\Delta\epsilon = 2.35 \times 10^3$ ($Mb^+ - Mb$). [i] At 560 nm, $\Delta\epsilon = 8.4 \times 10^3$ ($Mb - Mb^+$) and 1.4×10^2 ($Hr^+ - Hr$). [j] Rapid change at λ = 475 nm on adding azide to product, $\Delta\epsilon = 1.5 \times 10^3$ ($Mb^+N_3^- - Mb^+$). [k] Slow change at λ = 475 nm on adding azide to product, $\Delta\epsilon = 2.9 \times 10^3$ ($Hr^+N_3^- - Hr^+$). [l] At λ = 555 nm, $\Delta\epsilon = 7.3 \times 10^3$ ($Hb - Hb^+$) and 1.4×10^2 ($Hr^+ - Hr$).

[1] Abbreviations used: Hr, hemerythrin; Mb, myoglobin; Hb, hemoglobin all in Fe(II) form; Met represented by superscript +; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

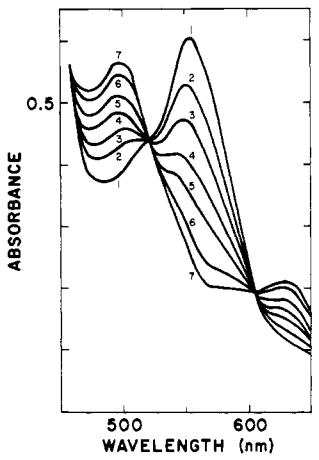

FIGURE 1: Spectra at various times after adding *T. pyroides* methemerythrin (0.28 mM) to sperm whale deoxymyoglobin (0.05 mM) at pH 7.0 and T = 25 °C. [Tris] = 0.05 M; [Na_2SO_4] = 0.05 M. Curves indicate (1) 1, (2) 6, (3) 10, (4) 15, (5) 20, (6) 30, and (7) ~100 min after reaction initiation.

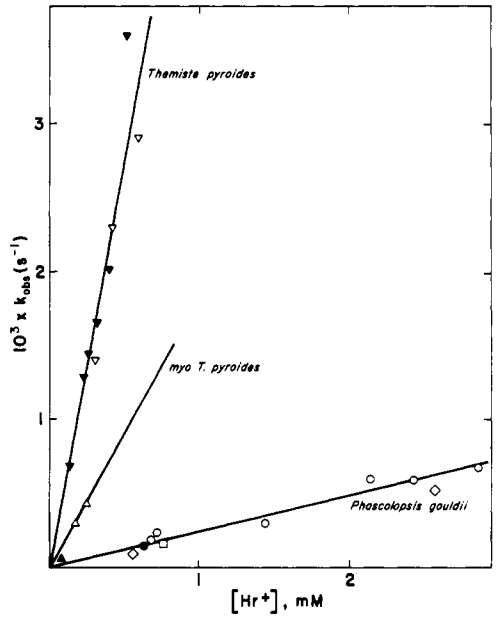

FIGURE 2: Plot of $10^3 \times k_{obsd}$ (s^{-1}) vs. [Hr^+] (mM) at pH 6.3, 7.0, and 8.2, I = 0.2 M, and T = 25 °C. With *P. gouldii* and horse heart myoglobin: (○) pH 6.3; (◇) pH 8.2; (●) purified sperm whale myoglobin, pH 6.3; (□) sperm whale, I = 1.35 M, pH 6.3. With *T. pyroides* and sperm whale myoglobin: (▲) monomer, pH 6.3; (△) monomer, pH 7.0; (▼) octamer, pH 6.3; (▽) octamer, pH 7.0.

reaction over 16 h at pH 6.3 or 8.2 when the proteins were separated by dialysis tubing. Puncturing the dialysis bag allowed the proteins to mix and the normal reaction rate was then observed. There was little effect of increasing the ionic strength from 0.2 M to 1.35 M or changing the pH from 6.3 to 8.2 on the rate of the reaction (Figure 2). At only pH 8.2 an occasional run (about one in four) gave kinetic spurious behavior (faster or non-first-order) for reasons which we are unable to explain. It is clear, however (Figure 2), that there is little change in the rate over pH 6.3. The methemerythrin azide adduct showed no reaction with deoxymyoglobin at pH 6.3 over 20 h. Addition of oxygen slowed reaction 1. Oxymyoglobin was oxidized to the met form by *P. gouldii* methemerythrin with second-order rate constants of 0.06 M^{-1} s^{-1} (pH 6.3) and 0.09 M^{-1} s^{-1} (pH 8.2).

There were problems in obtaining reproducible and clean kinetics, but not the stoichiometry, with reaction 2. This might be attributable to seepage of traces of oxygen and slight denaturing of hemoglobin at 25 °C over the hours of reaction necessary for completion. More likely the problems result from the tetrameric character of hemoglobin, with different reactivity and redox potentials for the 2α and 2β chains (Antonini & Brunori, 1971). It can be stated, however, that the reaction is slower than with myoglobin and a rate constant ~0.1 M^{-1} s^{-1} at pH 6.3 can be estimated for reaction of *P. gouldii* methemerythrin with human deoxyhemoglobin.

Discussion

When methemerythrin was added to deoxymyoglobin, there was no rapid initial spectral change, the subsequent kinetic curves were uniphasic and the final stoichiometry corresponded to reaction 1. Since the parameters $-d(Mb)/dt \equiv +d(Mb^+)/dt$ are being measured, these facts indicate that the reduction of one iron(III) of the pair in each subunit of hemerythrin is rate determining and that this event is followed rapidly by a second reduction. Once this has occurred in one of the eight subunits of the octameric protein, it appears that this has little influence on the behavior of the remaining subunits of the octamer. This conclusion is supported by the experiment using the monomeric form of hemerythrin, namely, metmyohemerythrin from the muscles of *T. pyroides*. This shows a similar rate constant (Figure 2) to that for the octameric form (calculating the latter on the basis of the molecular weight for the monomer). It was only possible to study a limited pH range (pH 6.3–8.2) because of the instability of the hemerythrin outside this range over the relatively long reaction times. There is a small effect of pH and ionic strength on the rate. The methemerythrin derived from *T. pyroides* is some 20 times more reactive than that from *P. gouldii*, a relative reactivity which we have found displayed toward inorganic reactants, although rarely as pronounced. The primary structure of *T. pyroides* myohemerythrin (Klippenstein et al., 1976) differs from the coelomic hemerythrin of *T. pyroides* (Ferrell & Kitto, 1971) in 60 residue positions, whereas the coelomic hemerythrin of *T. pyroides* and *P. gouldii* (Klippenstein, 1972; Klippenstein et al., 1968) differs in only four positions. Most, if not all, of the iron-coordinated ligands are the same, however, in all species (Stenkamp et al., 1976b, 1978) and this must be paramount in defining reactivity. It is interesting in this context that *P. gouldii* has shown no signs of multiphasic kinetic behavior in any of the reactions that we have studied, even though it is known that it contains a number of variants, differing in five amino acid interchanges (Klippenstein, 1972).

The reaction was shown to involve directly the two proteins, or at least was not one that was mediated by small molecular weight impurities, since separation of the two proteins by a dialysis bag suppressed any reaction. This approach has been previously utilized to show the absence of mediators in the ferricytochrome *c*–deoxymyoglobin reaction (Wu et al., 1972).

The only fourfold decrease in rate when deoxy was replaced by oxymyoglobin (in the presence of 0.24 mM O_2 when the concentration of deoxy will be reduced to less than 1% of the oxy) indicates an intrinsic reactivity for oxymyoglobin. Distinctive rate constants have also been noted for reaction of deoxy- and oxymyoglobin with ferricytochrome *c* (Ataullakhanov et al., 1976; Atanasov et al., 1977).

The details of electron transfer between metalloproteins are in a very speculative stage at present. Some general rules and observations have been outlined in a recent penetrating review (Moore & Williams, 1976). For the simpler inorganic redox reagents, it is clear that there are a variety of mechanistic pathways which can be used by the protein (Wherland & Gray, 1977) and in general there appears to be no real obstacle

in moving electrons from or to the protein. The transfer of electrons from one protein to another, however, poses a number of difficult questions. Myoglobin has its one heme group embedded in a hydrophobic pocket which reaches to the surface of the protein (Antonini & Brunori, 1971). It appears reasonable to use the polar edge of the heme, which is exposed at the surface, for electron transfer just as has been postulated as one of the available mechanisms for cytochrome *c* (Sutin, 1977; Salemme, 1977). The electron route to the irons in hemerythrin is even more difficult to map. In each monomer unit of octameric methemerythrin from *Themiste dyscritum*, one iron is attached to His-25, His-54, and Tyr-109, while the other iron is coordinated to His-73, His-77, and His-101. The irons are believed bridged by Glu-58 and Asp-106 (Stenkamp et al., 1976a,b, 1978). The histidines-73, -77, and -101 are in the C and D helical regions and these are exposed on the outside of the octamer. His-25 and -54 are in helices A and B and these are heavily involved in contacts with other units of the octamer. It can be estimated that the edge of His-73 is about 2 Å from the outside surface of helix C (Ward & Hendrickson, 1977). Electrons could be transferred through this imidazole "pump". Once the favored iron is reduced it may easily transfer the electron to the neighboring bridged iron and then be reduced again. The second reduction must, however, be faster than the first. The distance between the iron of the myoglobin and either iron of the hemerythrin, even in the most favorable relative juxtapositions, must be ~15 Å, about a maximum distance for electron transfer even within a good conducting medium (Moore & Williams, 1976). This, combined with small effective contact areas of each protein, compared with the total surface areas, leads to a reaction rate constant many orders of magnitude less than that of diffusion controlled. Very interestingly, there is little difference in the rate of reaction of the octameric compared with the monomeric form of hemerythrin so that the mode of electron transfer appears similar. The eight units of hemerythrin have a square antiprism arrangement with a 20-Å square hole (Ward et al., 1975; Stenkamp et al., 1976b). Apart from interactions with myoglobin on the outside of the octamer, the ellipsoid myoglobin (~22 × 12.5 Å) could be accommodated within the square, although this does appear unlikely.

The bimolecular rate constant for electron transfer between two molecules can be calculated provided a number of parameters of the system are known (Marcus, 1964; Hopfield, 1974; Jortner, 1976). One of these parameters is the distance of closest approach and, since this can be estimated fairly convincingly for cytochrome *c*, it has been possible to estimate the rate constant for a number of redox reactions of this protein with other proteins or small molecules and very good agreement with the experimental values has been noted (Chien et al., 1978). Since the reaction distances are so uncertain with the present system, a similar type of calculation is not feasible. However, the reaction must be of the outer sphere type so that we can apply the theory of Marcus (1964) as it relates to cross reactions (Marcus & Sutin, 1975). The approach ignores a number of factors (Wherland & Gray, 1977) but has been used successfully to rationalize the rate constants for reaction between a number of proteins (Sutin, 1977; Wherland & Pecht, 1978). In the simplest form, the rate constant k_{12} for the cross reaction (eq 1) is related to k_{11} and k_{22} (rate constants for self-exchange of hemerythrin and myoglobin, respectively) and K_{12} (the equilibrium constant for eq 1 by expression 3. The value obtained[2] for k_{11} ~10^{-3} M^{-1} s^{-1} for self-exchange of *T. pyroides* and ~10^{-5} for *P. gouldii* must be considered as very approximate ones, but they do indicate that electron transfer between the oxidized and reduced forms of hemerythrin is very slow, comparable to that estimated for ferredoxins (Rawlings et al., 1977). Significantly, both systems lack the excellent electron-conducting porphyrin ring. The reduction of methemerythrin by deoxymyoglobin is in striking contrast to that by dithionite. With the inorganic reductant, the reaction is multiphasic and an intermediate species containing Fe(II) and Fe(III) has been characterized (Harrington et al., 1978).

$$k_{12}^2 = k_{11}k_{22}K_{12} \quad (3)$$

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[2] We can assess a value for k_{22} of ~6 M^{-1} s^{-1} by applying relation 3 to the reaction between ferricytochrome *c* and deoxymyoglobin (Ataullakhanov et al., 1976). For this system, at pH ~7, I = 0.1 M, and T = 20–25 °C, k_{12} = 2.2 × 10^3 M^{-1} s^{-1}; K_{12} = 2.5 × 10^3 and k_{11} (the self-exchange rate constant for cytochrome *c*) = 3 × 10^2 M^{-1} s^{-1} (Gupta, 1973; Gupta et al., 1972). For reaction 1, K_{12} is estimated as ~10^3 M^{-1} from the extent of the reaction and by use of $E°_{mean}$ ~ 250 mV for hemerythrin from considerations of chemical reactivity.

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Functional Properties of Human Hemoglobin Bound to the Erythrocyte Membrane†

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ABSTRACT: Studies are presented which deal primarily with the ligand binding kinetic properties of human oxyhemoglobin bound to the erythrocyte membrane. Static 90° relative light scattering measurements are also presented as a necessary preliminary to the functional studies. The light scattering measurements suggest that the dimer of oxyhemoglobin binds to the cytoplasmic surface of the membrane. Binding was apparently noncooperative with a constant of about 4 μM in dimer at pH 6, in 5 mM phosphate buffer at 23 °C. Further evidence for enhanced formation of the oxygenated dimer was obtained from kinetic measurements where oxygen was rapidly removed in the stopped-flow and the kinetics of CO binding studied. A substantial increase in the proportion of rapid CO binding component was observed with increasing ghost concentration. Complete reversibility of the increased fraction of rapid CO binding component was demonstrable upon addition of the enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PD) after addition of oxyhemoglobin. This result may indicate that the oxygenated dimer binds reversibly to band 3 since G3PD has been shown to bind to this membrane protein (Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9176). Oxygen release measurements in the absence and presence of CO indicate diminished cooperativity, as expected if the oxygenated dimer is stabilized. However, the initial rate constant for oxygen release from the β chain of the bound dimer was found to be about 20 times slower than in solution.

The interaction of hemoglobin with the erythrocyte membrane is interesting, both as a model system to study how cytoplasmic components may interact with a plasma membrane and as part of the current effort to develop a more integrated physical description of the human erythrocyte. Detailed studies of this interaction have been presented by Fischer et al. (1975) and Shaklai et al. (1977 a,b). First, and most importantly, it was demonstrated that hemoglobin binding was reversible and largely electrostatic in nature (Fischer et al., 1975). Fluorescence quenching studies (Shaklai et al., 1977a,b) showed the stoichiometry and sidedness of binding and values were obtained for the binding constant. The number of sites involved corresponded to the number of band 3 polypeptides (the predominant, integral membrane protein of the human erythrocyte), and competition experiments with G3PD,[1] an enzyme known to bind to band 3 (Yu & Steck, 1975a,b), offered further evidence for the location of the binding site.

As a consequence of these initial physical characterizations, it was natural to inquire about the functional properties of membrane-bound hemoglobin since the activities of certain enzymes have been altered upon binding (Strapazon & Steck, 1976; Karadsheh & Uyeda, 1977). We elected to investigate the functional properties of membrane-bound hemoglobin using kinetic techniques. Besides the kinetic studies, we also present some new static 90° relative light scattering measurements on the binding of oxyhemoglobin to the inner surface of the membrane. This technique allows measurement of oxyhemoglobin binding over a wide heme concentration range and was needed for comparison with the earlier fluorescence studies.

Materials and Methods

Hemoglobin and unsealed ghosts were prepared as previously described (Shaklai et al., 1977a,b). The unsealed ghosts were equilibrated once with 50 mM NaCl at pH 8 to release

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[1] Abbreviation used: G3PD, glyceraldehyde-3-phosphate dehydrogenase.

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