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Electron-transfer self-exchange kinetics of trimethylphosphine horse-heart myoglobin

Christian Brunel, Arnaud Bondon and G. Simonneaux

Laboratoire de Chimie Organométallique et Biologique, Université de Rennes, Rennes (France)

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Electron self-exchange has been measured by an NMR technique for horse-heart myoglobin. The rate is $3.1 \cdot 10^3$ M^{-1} s^{-1} at 23°C in 0.1 M phosphate at pH 6.9. The rate was weakly dependent on ionic strength up to 0.7 M in added KCl ($3.9 \cdot 10^3$ M^{-1} s^{-1}). The enthalpy of activation was 12.1 ± 0.5 kcal mol^{-1}, and the entropy of activation was -1.2 ± 0.5 cal mol^{-1} deg^{-1}. Analysis of the data in terms of the Marcus theory gives a reorganization energy, λ, for self-exchange of 1.6 eV mol^{-1}.

Introduction

Long-range electron transfer is an essential component of biological systems, playing an important role in respiration and photosynthesis [1]. Much current research is centered around probing what factors control the rates of electron transfer [2] and the preparation of multi-site redox proteins allows such studies [3,4]. In recent years, mitochondrial cytochrome *c* has been one of the proteins most widely used as the study system [5–8]. Even though hemoglobin and myoglobin are not directly involved in electron transfer reactions, an understanding of the mechanism of a self-exchange reaction involving these proteins is important in the understanding of the overall picture of electron transfer processes in heme proteins. Fewer studies of redox reactions of the oxygen carriers, hemoglobin and myoglobin [9–11], have been undertaken, in part, because suitable reduced and oxidized states have not been available [12]. These studies have focused on the oxidation-reduction pathways of myoglobin (or hemoglobin) with a variety of inorganic reactants. For most of these systems, and especially near physiological pH, the electron-transfer mechanism is almost certainly of the outer-sphere type. However, the reported self-exchange rate constants of myoglobin, calculated from Marcus theory with inorganic redox couples, span a wide range [13,14], thought to reflect different mechanisms and distances for electron transfer from different reagents. Furthermore the experimental self-exchange process was not determined in these systems.

Several considerations make horse heart myoglobin an excellent candidate for such determination. For instance, a large body of functional data is already available [15–17] and the three-dimensional structure of the met-form has been determined [18]. In addition, trimethylphosphine (PMe_3) may serve as ligand to both the ferric and ferrous heme of myoglobins [19,20]. Among the many interesting features of this complexation is the presence of a well resolved upfield-shifted methyl-group resonance of PMe_3, coordinated to the metal atom in both oxidation states in the NMR spectra. Consequently, this protein is particularly amenable to study via NMR techniques. The subject of this report is the non-physiological electron transfer study in trimethylphosphine horse heart myoglobin between the two redox states, Fe II and Fe III. The electron transfer self-exchange rate constant has been measured as a function of ionic strength, temperature and pH. The reorganization energy has been calculated from the rate constant at a given ionic strength and temperature.

Material and Methods

Horse-heart myoglobin and sperm whale myoglobin were purchased from Sigma Chemicals and purified on a CM-52 ion-exchange column [21]. Trimethylphosphine metmyoglobin samples were preparated under argon by adding a 3-fold excess of PMe_3 to 2 mM metmyoglobin solutions in 0.1 M phosphate buffer, pH

Correspondence: G. Simonneaux, Laboratoire de Chimie Organométallique et Biologique, URA CNRS 415, Université de Rennes 1, Campus de Beaulieu, Rennes, France.

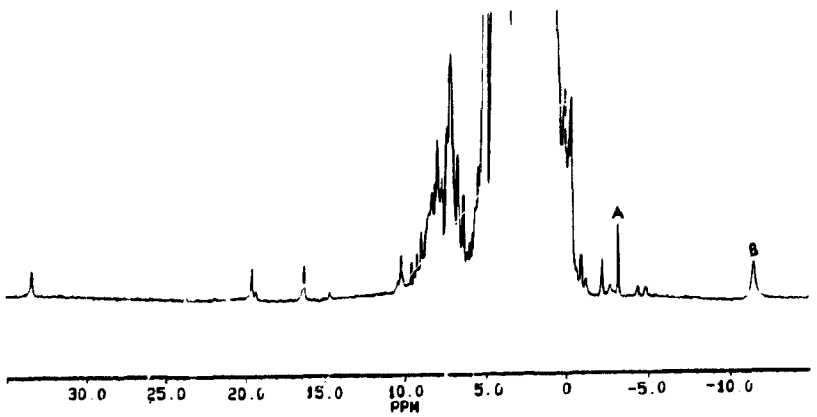

Fig. 1. The 300 MHz ^{1}H-NMR spectrum of a mixture of horse-heart myoglobin metMbPMe$_3$/MbPMe$_3$ in the ratio 70/30 in D_2O at 23°C (pH 6.9). Assigned resonances: A, Fe(II)PMe$_3$; B, Fe(III)PMe$_3$.

6.9 in D_2O; pH values were uncorrected for the isotope effect. For the experiment utilizing a mixture of metMbPMe$_3$/MbPMe$_3$, the appropriate equivalent of sodium dithionite was added in D_2O. Samples were run in $\mu = 0.1$ M phosphate buffer (pH 6.9), with added KCl. Proton NMR spectra were recorded on a Bruker AC 300 P spectrometer and a Bruker AM 300 WB spectrometer in a temperature-regulated probe (CRMPO, Rennes). Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through the residual water resonance.

For calculation of the rate constants, a 180°-τ-90° sequence was used. A delay time (τ) of 0.002–1 s was used as the exchange period between the non-selective 180° pulse and the 90° detection pulse. Each measurement had a series of 20 different τ values. Data were taken in blocks of 32 scans with two dummy scans between each block. The spectral width was 20.8 kHz.

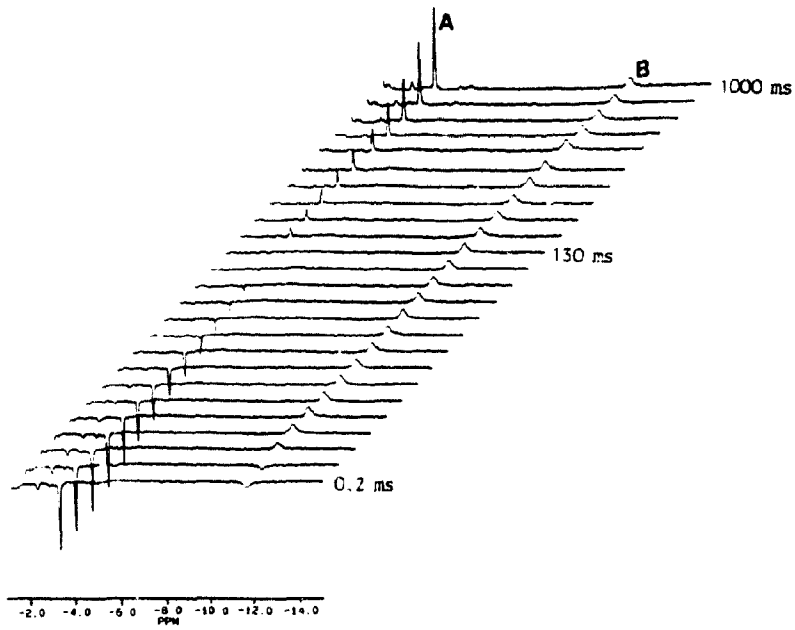

Fig. 2. Partially relaxed ^{1}H NMR spectra of horse heart myoglobin, as a function of τ in a 180°-τ-90° T_1 inversion-recovery experiment. Assignment of the methyl group resonances: A, Fe(II)PMe$_3$; B, Fe(III)PMe$_3$.

The data were analyzed by using the method previously reported by Gupta and Mildvon [22]. For the reduced-state methyl resonance of PMe_3 at −3.3 ppm, the nonselective T_1 was determined as 370 ± 10 ms in the absence of metmyoglobin. The measurements were found reproductible to better than 10%. A selective 180° pulse, inverting only the peak of interest, was also used. As expected, this gives shorter T_1 values than the method in which all the peaks are inverted, due to the contribution of the nuclear Overhauser effect to relaxation of the magnetisation. In this case, the selective T_1 for the reduced-state methyl resonance of PMe_3 was determined as 145 ± 10 ms, in the absence of metmyoglobin. The rate cons ants calculated from the two methods were the same within experimental error. Since the two redox states of horse-heart myoglobin PMe_3 are characterized by different spectra, observed resolved resonances included the three-methyl resonance of the phosphine, upfield of 0 ppm from both the ferrous state and the ferric state in the mixed sample [20]. The spin-lattice relaxation time T_1 (nonselective pulse) was 370 ms and 4 ms in the completely reduced and oxidized states, respectively, for the phosphine protons. The ratio of oxidized to reduced protein was determined from the integrated areas (PMe_3) of the two forms of the complexed protein in the NMR spectrum. The relaxation rates reported here were measured in the Fe (II) protein and assume that the lifetime in the oxidized state is long compared to the spin-lattice relaxation time in this state [22]. The rate constants determined in this study were found to be independent of protein concentration within experimental error (range of protein concentrations: 2–6 mM).

Results

A mixture of oxidized and reduced trimethylphosphine horse-heart myoglobin undergoes electron transfer in the slow exchange time limit, where resonances from both the oxidized and reduced protein are seen clearly in a mixture of the two proteins (Fig. 1). In particular, both the PMe_3 methyl resonances from the oxidized and reduced proteins are seen in the upfield region of the NMR spectrum. Inversion recovery techniques were used to measure the rate constant for electron exchange [7,22] (Fig. 2). In 0.1 M potassium phosphate buffer (pH 6.9) and 23°C, the bimolecular rate constant for self-exchange for horse-heart myoglobin is $3.1 \cdot 10^3$ M^{-1} s^{-1}.

The ionic strength dependance of the myoglobin self-exchange rate is shown in Fig. 3. As seen from the Fig. 3, the rate constants for horse-heart myoglobin increase weakly with ionic strength. For example, the rate under the previous conditions but, with added KCl to 0.7 M, was $3.7 \cdot 10^3$ M^{-1} s^{-1}. In order to follow the

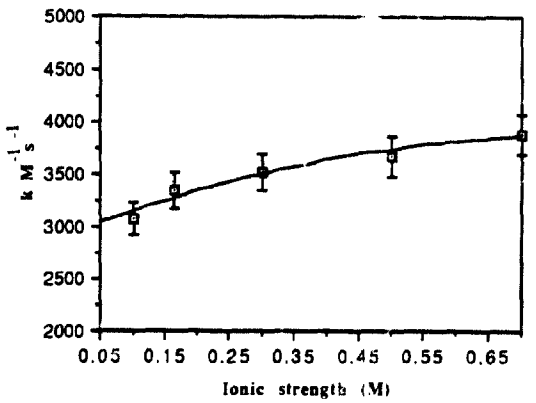

Fig. 3. The ionic strength dependance of the myoglobin electron-transfer self-exchange rate constant (pH 6.9, 23°C).

ionic strength dependance of the rate of a reaction between two large proteins with a dipole moment, we used the van Leeuwen approach [23]. For instance, sperm whale myoglobin has a dipole moment of 170 D [24]. The method also assumes that electron transfer occurs at the partially exposed heme edge. An extrapolation to infinite ionic strength of a plot of the ionic strength dependance of the self-exchange rate between myoglobins versus $f(\kappa)$ (κ is $0.329\,\mu^{1/2}$) yields $k_{int} = 5 \cdot 10^3$ M^{-1} s^{-1}. The method also allows the determination of an interaction energy, w_r, of 0.30 kcal mol^{-1} for the two myoglobins in a heme edge-to-heme edge geometry.

The temperature dependance of the self- exchange rate constant was investigated over the range 23°–42° in 0.1 M phosphate (pH 6.9) (Fig. 4). The values of ΔH^* and ΔS^* obtained from an Eyring plot at 0.1 M ionic strength and 2 mM protein concentration are 12.1 ± 0.5 kcal mol^{-1} [12] and −1.2 ± 0.5 cal mol^{-1} deg^{-1}, respectively ($\Delta G^* = 12.5 \pm 0.5$ kcal, 25°C).

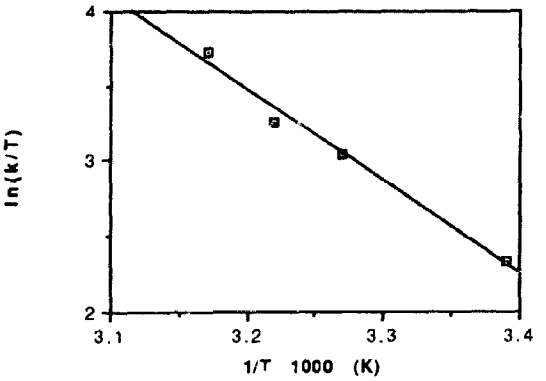

Fig. 4. Eyring plot of the myoglobin electron-transfer self-exchange at μ = 0.1 M, [Mb] = 2 mM.

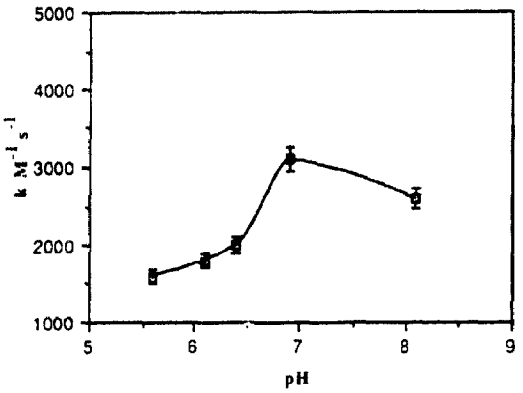

Fig. 5. pH dependence of the electron-transfer rate at low ionic strength (0.1 M, 23°C).

The rate at low ionic strength (0.1 M, 23°C) varies with pH, in a manner shown in Fig. 5. One obtains a rate up from $k = 1.6 \cdot 10^3$ M^{-1} s^{-1} (pH 5.6) to $3.1 \cdot 10^3$ M^{-1} s^{-1} (pH 6.9) which falls off to $k = 2.5 \cdot 10^3$ M^{-1} s^{-1} with increasing pH to 8.1. Myoglobin is capable of transferring electrons at acidic and basic pH with a small decrease in both cases.

For sperm whale myoglobin under the same conditions, at 0.1 M ionic strength (pH 6.9, 23°C), the self-exchange rate constant was slightly higher, $4.1 \cdot 10^3$ M^{-1} s^{-1}.

Discussion

Hemoproteins have a high affinity for phosphines [25–27]. We have shown that electron transfer in vitro between trimethyl phosphine myoglobin molecules of different oxidation states occurs with moderate rapidity. In general, long cytochromes (100–115 aminoacids) exchange electrons slowly (10^3–10^4 M^{-1} s^{-1}) while short cytochromes (80–90 aminoacids) do so quickly (10^6–10^7 M^{-1} s^{-1}) [6,7,28–30]. The rate constants for the proteins studied here are remarkably similar, at low ionic strength, to those previously reported for horse-heart cytochrome *c* ($5.4 \cdot 10^3$ M^{-1} s^{-1}) [7] and for cytochrome b_5 ($2.6 \cdot 10^3$ M^{-1} s^{-1}) [28]. It is unexpected that the rates exhibited by these proteins are so similar because the polypeptide chain of myoglobin, containing 153 aminoacid residues, is longer than the polypeptide chain of cytochrome *c* (103 amino acids) and the trypsin-solubilized form of cytochrome b_5 (82 amino acids). This indicates that the rate of self-exchange electron transfer in myoglobin is affected by other factors.

Electrostatic interactions are obviously important for self-exchange transfers in proteins. The overall charge calculated is +3.5 at pH 6.8 for ferromyoglobin [13,31] and is +6 at pH 7 for ferrocytochrome *c* [32]. When the protein molecules are charged, their distance of closest approach is limited, due to electrostatic repulsion. Consequently, charge effect may be more important in cytochrome *c* than in myoglobin at pH 7. As seen previously (Fig. 3), the increase is very low with horse-heart myoglobin and we conclude that the rate is weakly dependant on ionic strength over this range of salt concentration. Studies on electron self-exchange for cytochromes *c*-551 from *Pseudomonas aeruginosa* [29] and *Pseudomonas stutzeri* show similar behaviors [30]. In contrast, the rate constants for cytochrome b_5 [28] and cytochrome *c* [33] increase, by an order of magnitude, over the same range of ionic strength, as expected for a reaction between two similarly charged proteins.

Other contributing factors in myoglobin in electron transfer self-exchange include the percent of heme exposure [34], steric effect [7], dipole moment [7,23] and the orientation of heme propionate groups [35]. For example, it has been proposed for many years, that electron transfer in cytochromes occurs primarily near the exposed edge of the heme. The heme of myoglobin (18%) is far more exposed to solvent than that of cytochrome *c* (4%) [34]. This difference is numerically significant and leads to the prediction that the the myoglobin electron self-exchange rate should be substantially greater than that of cytochrome *c*. However, the measured rate constants for the two proteins are similar at $\mu = 0.1$ M and, in contrast, the value for the cytochrome *c* system is much larger than that for the myoglobin system, at high ionic strength. Consequently, this difference in the electrostatically-corrected rate constants is not, due primarily, to the difference in heme exposure [7,34].

It is, therefore, of interest to calculate the reorganization energy from the rate constant at a given ionic strength and temperature. This approach was recently reported by Dixon et al. [28]. The authors have obtained a value of 0.72 eV for cytochrome *c* and a value of 1.2 eV for cytochrome b_5. With the use of the Marcus formalism [2], the myoglobin self-exchange rate constant can be expressed as:

$$k_{el} = SK_a \nu_n \kappa_{el} \exp(-\Delta G_t^*/RT)$$

where S is the steric factor, K_a is the association constant for formation of the precursor state from the two myoglobins, ν_n is the nuclear frequency factor, κ_{el} is the probability of electron tunneling, and ΔG_t^* is the free energy of activation. The association constant K_a can be expressed as

$$K_a = 4\pi N r^2 \delta(r) \exp(-w_r/RT)$$

where N is the Avogadro's number, $r = 36.8$ Å [31] is

the sum of radii of the two proteins, $\delta(r)$ is usually taken as $\beta^{-1} = 1.11$ Å [28] and, with the use of van Leeuwen formalism, $\exp(-w_r/RT) = \ln(k_1/k_{inf})$ (w_r is 0.31 kcal mol^{-1} when $\mu = 0.1$ M). Given these values, we calculate $K_a = 6.72$ M^{-1}. Marcus and Sutin [2] have introduced the steric factor S to allow for the fact that there is an angular dependance of the electron transfer. A lower limit for the steric factor S is equal to the square of the ratio of the surface area of the exposed heme edge to the surface area of the protein. The surface of myoglobin is 2% heme [34]. Using this assumption, and an enhancement factor of 5[28], we have estimated the value of S as 0.010. $\nu_n \kappa_{el}$ can be expressed as

$$\nu_n \kappa_{el} = 10^{13} \exp(-\beta(d - d_0))$$

where d is the closest heme-heme distance, $d_0 = 3$ Å and $\beta = 0.9$ Å^{-1} are the standard values [2,28]. Using the hemoglobin-cytochrome b_5 model, previously reported by Poulos and Mauk [36], for myoglobin-myoglobin complex, we estimate a (similar) value of $d = 7$ Å for the closest approach in the heme edge-to-heme edge geometry. This analogy is made on the basis that hemes, which are not covalently attached to the polypeptide chains as in hemoglobin, myoglobin and cytochrome b_5, are oriented such that the heme propionate groups are located on the protein surface. In addition, the similarity in the heme exposure of all three proteins is striking: Mb 18%, Hb_α 14%, Hb_β 20% and cytochrome b_5 23% [34]. Therefore, we calculate a value for $\nu_n \kappa_{el}$ of $2.7 \cdot 10^{11}$ s^{-1} and, with the above values for K_a, S and $\kappa\nu$, the value of ΔG_r^* is 9.20 kcal mol^{-1}. The reorganization energy λ can obtained through application of the following relationships, according to the Marcus formalism:

$$\Delta G_r^* = (\lambda/4)(1 + \Delta G^{0'}/\lambda)^2$$

$$\Delta G^{0'} = \Delta G^0 + w_p - w_r$$

where ΔG_r^* is the free energy of activation that is related to λ, to ΔG^0, the free energy change of the reaction, and to the work of bringing the reactants (w_r) or products (w_p) to the mean separation distance in the electron transfer complex. For self-exchange reactions, ΔG^0 is zero and $w_p = w_r$. Therefore, the energy of reorganization can be expressed as: $\lambda = 4\ \Delta G_r^*$ and the reorganization energy for the self-exchange reaction of myoglobin is 1.6 eV. Values of β in the range of 0.9–1.4 Å^{-1} [37] have also emerged from studies of long-distance electron transfer reaction in ruthenium-modified myoglobin. (With $\beta = 1.4$ Å^{-1}, for example, the associated reorganization energy is 1.4 eV.) Although β varies with the system [34], we have chosen $\beta = 0.9$ Å^{-1} in the present work, since this value is similar to most other estimates of this parameter [28,38].

Our reorganization energy for myoglobin can be compared to that for cytochrome c, 0.72 eV and for cytochrome b_5, 1.2 eV, which were recently reported by Dixon and Mauk [28]. The origin of the greater value for myoglobin may be due, primarily, to the lower relative stability (greatest flexibility) of the heme pocket for horse-heart myoglobin, compared to those of cytochrome b_5 and cytochrome c. It is now well established that the nature of the heme interactions with proteins is not sufficiently selective to force a single orientation of the heme group within the pocket of hemoproteins [39]. The heme moiety of cytochrome c is covalently linked by thioether bonds at the vinyl groups and co-ordinated by histidine and methionine side chains [40]. Accordingly, cytochrome c exhibits no major differences inside the heme pocket in the oxidized and reduced states [2]. In contrast, the heme binding of myoglobin is non-covalent [18] as in cytochrome b_5 [41,42], and, furthermore, in myoglobin, the sixth ligand (here the phosphine) is also non-covalently bound to the polypeptide chain. The reorganization energy, arising partially from changes in the metal ligand bond distances [2], may increase because it is possible that the iron-ligand distances in the two oxidation states are quite different. As an essential complement of this study, we recently reported the structures of two model phosphine complexes of iron(II) and iron(III) porphyrins [43,44]. The axial metal-phosphorus bond length in the ferric complex (2.350(1) Å) [44] is significantly longer than that observed in the analogous ferrous complex (2.284(1) Å) [43]. This observation is consistent with the suggestion that changes in the iron-ligand distances may result from the change in oxidation state of the iron atom of myoglobin. Our reorganization energy can be also compared with the intramolecular reorganization energies for electron transfer within ruthenium-modified myoglobins. Gray et al. have recently investigated a wide range of reaction-free energies that places the reorganization energy for ruthenium-modified myoglobin between 1.90 and 2.45 eV [38]. These values are slightly greater than our value, indicating that a similar analysis of intramolecular electron transfer reactions and bimolecular reactions may be applied [28,37,45,46].

We note that the values of ΔH^* and ΔS^* observed here are remarkably similar to those previously reported for electron transfer reactions between two metalloproteins [6]. The positive activation entropies for the self-exchange electron transfer reactions with myoglobin [6], azurin [47] and horse-heart cytochrome c [6] systems are noteworthy. This pattern may result from the extensive hydration of the proteins and, consequently, water rearrangement might play an important role in the activation process [6]. By contrast, the

values of ΔS^{*} observed with cytochrome b_5 are negative (−23 eu) [28] though the origin of this difference is not apparent at present.

A final comment concerns the estimation of the electron self-exchange rate of oxygen carriers (myoglobin or hemoglobin) between oxidized and reduced states. To our knowledge trimethylphosphine is the first ligand which allows suitable reduced and oxidized states and makes the experimental determination possible. The reported self-exchange rate constant of myoglobin, calculated from the Marcus theory with inorganic redox couples, varies from $5 \cdot 10^{-2}$ to $1.2 \cdot 10^{1}$ M^{-1} s^{-1} [13]. For example, from the kinetic of the reduction of myoglobin by $Fe(EDTA)^{2-}$ (EDTA, ethylenediamine tetraacetate), the self-exchange rate constant was calculated to be $1.26 \cdot 10^{-1}$ at pH 7 and 25°C. The large discrepency between the experimental measurements and the calculated values indicates that an estimation of the self-exchange rate constant of the metMb/deoxyMb system, based on the Marcus theory, is difficult, because this system requires a change in the coordination number upon reduction of metMb(H_2O) to deoxyMb. Accordingly, it was recently reported that modification of the hemes distal histidine by BrCN resulted in acceleration of the self-exchange rate of myoglobin, as compared with its native form. The self-exchange rate constant for the penta-coordinated modified metmyoglobin/deoxyMb system was evaluated to be $1 \cdot 10^{4}$ M^{-1} s^{-1}, based on the Marcus theory [14]. Extension of these studies to other oxygen carriers, such as hemoglobins, which are in progress in this laboratory, will provide a more definitive description of the various factors which govern the electron transfer.

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