Possible Role of the Iron Coordination Sphere in Hemoprotein Electron Transfer Self-Exchange: ^{1}H NMR Study of the Cytochrome *c*−PMe_3 Complex

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The rates of self-exchange electron transfer in the trimethylphosphine complex of cytochrome *c* have been measured by an NMR technique over a large range of ionic strengths. The rate constant is 1.56×10^4 M^{-1} s^{-1} at 23° C ($\mu = 0.34$ M) at pH 6.9. Dependence on ionic strength of the rate constant is treated by van Leeuwen theory. Extrapolation of the rate constant to infinite ionic strength gives a rate constant of 3.9×10^5 M^{-1} s^{-1}. This rate constant is compared with others reported for myoglobin and cytochrome b_5. The values for these systems range over 2 orders of magnitude with myoglobin−PMe_3 ≪ cytochrome b_5 < cytochrome *c*−PMe_3 < cytochrome *c*. Analysis of the data in terms of Marcus theory gives a reorganization energy, λ, for self-exchange of 0.75 eV mol^{-1} for cytochrome *c*−PMe_3. Substitution of Met-80 by PMe_3 appears to influence only weakly the rearrangement barrier to electron transfer.

Introduction

In recent years, considerable interest has been focused on the role of the protein in controlling the rate of electron transfer.[1] The kinetics of electron transfer between metalloproteins are controlled by numerous factors.[2] Among these factors, the reorganization energy, which is a key determinant of electron transfer rates of hemoproteins, can be determined directly from self-exchange experiments. Due to their simplicity, such experiments permit the study of bimolecular reactions in solution without the influence of the relative thermodynamic stability of the reactants and products.[3] The reorganization energy is comprised of energy changes that are necessary to reach the transition state by bond lengthening, compression and torsion, bond angle changes, and reorganization of solvent molecules.

Very recently, considerable progress has been made in understanding how the protein structure controls the transfer process, both from the experimental[1,4] and the theoritical[5] points of view. The question of the distance dependence of electron transfer rates in metalloproteins has been addressed by Gray[6] and Isied[7] using different hemoproteins labeled with ruthenium complexes. Thus the possibility of single versus multiple pathways has been discussed in protein electron transport. In these studies, myoglobin and cytochrome *c* serve as protein models. In a different approach, we present here the results of the study of the kinetics of the self-exchange electron transfer reaction of phosphine-complexed cytochrome *c*. These results are compared with those obtained from the study of the similar self-exchange electron transfer reactions of various hemoproteins, and their indirect implications for biological electron transfer in general are discussed.

In previous work, we have reported the results of self-exchange electron transfer kinetic studies on myoglobin and hemoglobin involving ligation of trimethylphosphine complexation in both ferrous and ferric states.[8] In that investigation, we noted that the application of the Marcus relationship to evaluate the reorganization energy values related to the self-exchange rate constants of the Fe(III)/Fe(II) couples yielded much larger values for reaction in which myoglobin was involved than for reactions in which native cytochrome *c* was involved. We proposed that the apparent differences in the electron-transfer behavior of these hemoprotein systems may be a reflection of differing degree of flexibility of the metal coordination sphere in the heme pocket of the two proteins. Such analysis was also recently suggested by Winkler, Gray, and co-workers[9] to account for the distant electronic coupling in ruthenium/zinc porphyrin derivatives of recombinant human myoglobins.

In the current study, we have completed[10] a thorough kinetic investigation of self-exchange reactions involving different hemoproteins using trimethylphosphine complexation of cytochrome *c*. The primary focus of this study is to determine whether the bimolecular electron transfer rates are largely dependant upon the flexibility of the redox states in a manner consistent with the number of valence bonds between the heme and the protein. For the present study, the availability of previous results obtained with native cytochrome *c*[11,12] and cytochrome *b5*[13,14] makes possible a comparison between the

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(1) (a) *Electron Transfer in Inorganic, Organic and Biological Systems*; Bolton, J. R., Mataga, N., McLendon, G., Eds.; Advances in Chemistry Series 228; American Chemical Society: Washington, DC, 1991. (b) Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369.
(2) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265.
(3) Marcus, R. A. *Angew. Chem., Int. Engl. Engl.* **1993**, *32*, 1111.
(4) (a) Karas, J. L.; Lieber, C. M.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 599. (b) Therien, M. J.; Chang, J.; Raphael, A. L.; Bowler, B. E.; Gray, H. B. *Struct. Bonding* **1991**, *75*,109. (c) Moser, C. C.; Keske, J. M.; Warncke, K.; Farid, R. S.; Dutton, P. L. *Nature* **1992**, *355*, 796.
(5) (a) Beratan, D. N.; Onuchic, J. N.; Betts, J. N.; Bowler, B. E.; Gray, H. B. *J. Am. Chem. Soc.* **1990**, *112*, 7915. (b) Beratan, D. N.; Betts, J. N.; Onuchic, J. N. *Science* **1991**, *252*, 1285. (c) Regan, J. J.; Risser, S. M.; Beratan, D. N.; Onuchic, J. N. *J. Phys. Chem.* **1993**, *97*, 13083. (d) Siddarth, P.; Marcus, R. A. *J. Phys. Chem.* **1993**, *97*, 13078.
(6) Winkler, J. R.; Nocera, D. G.; Yocom, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1982**, *104*, 5798.
(7) (a) Isied, S. S.; Worosila, G.; Atherton, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 7659. (b) Moreira, I.; Sun, J.; Cho, M. O. K.; Wishart, J. F.; Isied, S. S. *J. Am. Chem. Soc.* **1994**, *116*, 8396.
(8) (a) Brunel, C.; Bondon, A.; Simonneaux, G. *Biochim. Biophys. Acta* **1992**, *1101*, 73. (b) Simonneaux, G.; Bondon, A.; Brunel, C. *J. Am. Chem. Soc.* **1994**, *116*, 11827.
(9) Casimiro, D. R.; Wong, L. L.; Colon, J. L.; Zewert, T. E.; Richards, J. H.; Chang, I. J.; Winkler, J. R.; Gray, H. B. *J. Am. Chem. Soc.* **1993**, *115*, 1485.
(10) Legrand, N.; Bondon, A.; Simonneaux, G.; Schejter, A. *Magn. Reson. Chem.* **1993**, *31*, S23.

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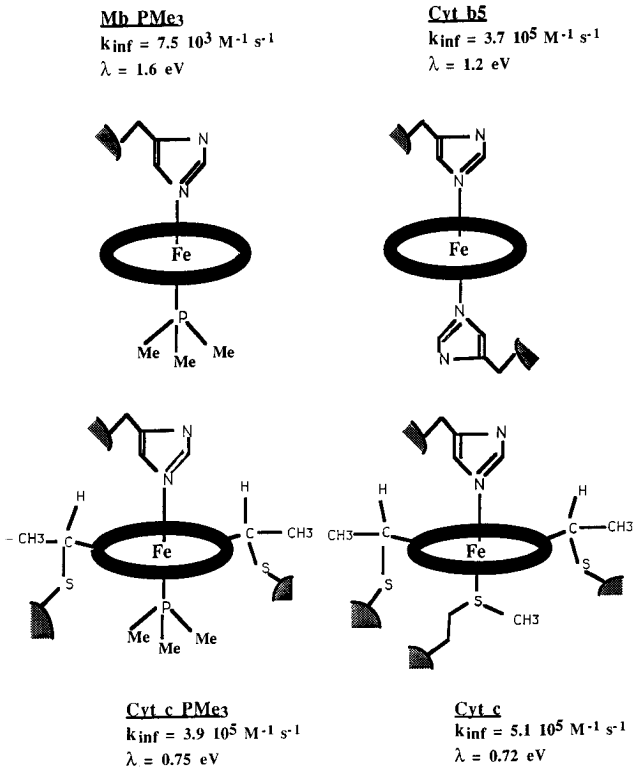

Figure 1. Schematic iron coordination sphere displaying the heme−protein bonds, calculated rate constants at infinite ionic strength k_{inf} and reorganization energies λ for myoglobin−PMe_3, cytochrome b_5, cytochrome c−PMe_3, and native cytochrome c.

four possibilities (Figure 1) and thus provides a test of the veracity of this approach.

Experimental Section

Chemicals. Horse heart cytochrome *c* type III from Sigma Chemical Co. was purified on a CM-52 ion-exchange column.[15] Trimethylphosphine cytochrome *c* samples were preparated under argon by adding a 3-fold excess of PMe_3 to 2mM cytochrome *c* solutions in 0.1 M phosphate buffer, pH 6.9 in D_2O. pH values were uncorrected for the isotope effect. For the experiment utilizing a mixture of cytochrome c−Fe(III)−PMe_3/cytochrome *c*−Fe(II)−PMe_3, the appropriate equivalent of sodium dithionite was added in D_2O. Samples were run in 0.1 M phosphate buffer, pH 6.9, with added KCl. Imidazole (or cyanide) UV−visible samples were preparated under argon by adding a large excess of the ligand to cytochrome *c* solutions in 0.1 M phosphate buffer, pH 6.9 in D_2O. UV−visible spectra were recorded on an Uvikon 941+ spectrophotometer.

Kinetics. Proton NMR spectra were recorded on a Bruker AC 300 P spectrometer in a temperature regulated QNP 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 nonselective 180° pulse and the 90° detection pulses. 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. The data were analyzed by using the method previously reported by Gupta and Mildvan.[11a] Since the two redox states of cytochrome *c*−PMe_3 are characterized by different spectra, observed upfield shifted resonances included the three-methyl resonance of the phosphine from both the ferrous state and the ferric state in the mixed sample. For the methyl resonance of PMe_3, the spin−lattice relaxation times T_1 (nonselective pulse) were 370 and 4 ms in the completely reduced and oxidized states, respectively. The measurements were reproducible to better than 10%. 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. A small signal appears also as a doublet in the spectrum (see Figure 3A). This is probably due to to the phosphine complexation of a minor form of cyt *c*. Corrections were applied for the small amounts of native cytochrome *c* and for the minor form. The relaxation rates reported here were measured in the Fe (II) protein, using the phosphine signal. It is assumed that the lifetime in the oxidized state is long compared to the spin−lattice relaxation time in this state. Since the heme iron in the oxidized state is paramagnetic, the phosphine methyl protons have a short relaxation time (4 ms) in this state. In mixtures containing millimolar levels of each of the two oxidation states, the lifetime of the methyl group in either state is of the order of T_1^{Red} ($\gg T_1^{Ox}$). Hence the method is applicable. The rate constants determined in this study were found to be independent of protein concentration within experimental error (range of protein concentrations: 2−4 mM).

Treatments of Electrostatic Interactions. The theory of the dependence of reaction rates on ionic strengths has been developped by van Leeuwen.[16] We also use this approach which has been recently proven very effective at the high ionic strength needed for these studies.[13,14,17] The theory recognizes net charges (Z) and dipole moments (D) of the protein molecules according to eqs 1 and 2. Z_{red}

$$\ln(k_1/k_\infty) = -\{Z_{ox}Z_{red} + (ZD)(1 + \kappa R) + (DD)(1 + \kappa R)^2\}(q^2/4\pi\epsilon\epsilon_o k_B RT)\, f(\kappa)$$

$$ZD = (Z_{ox}D'_{red} + Z_{red}D'_{ox})/qR$$

$$DD = D'_{ox}D'_{red}/(qR)^2 \quad (1)$$

$$f(\kappa) = [1 - \exp(-\kappa R)]/(\kappa R(1 + \kappa R/2)) \quad (2)$$

and Z_{ox} are the net charges on ferro and ferricytochrome *c*. D'_{red} and D'_{ox} are the components of the dipoles of the proteins through their respective exposed heme edge. $R = R_1 + R_2$ is the sum of the radii of the two proteins. $\kappa = 0.329\mu^{1/2}$. k_1 is the rate constant at a given ionic strength. k_∞ is the rate constant at infinite ionic strength. q is the elementary charge ($q = 1.6 \times 10^{-19}$ C). ϵ is the dielectric constant of water (80). ϵ_o is the dielectric permitivity (8.85418×10^{-12}). k_B is Boltzmann's constant (1.3807×10^{-23} J K^{-1}).

Results

Previous results indicate that the iron−sulfur bond is weak in the oxidized state of cytochrome *c* and the axial methionine is easily displaced by various ligands, such as cyanide,[18−20] pyridine,[21a] and imidazole.[21b] In contrast, it was recognized

(11) (a) Gupta, R. K.; Mildvan, A. S. *Methods Enzymol.* **1978**, *54*, 171. (b) Gupta, R. K.; Koenig, S. H.; Redfield, A. G. *J. Magn. Reson.* **1972**, *7*, 66. (c) Gupta, R. K. *Biochim. Biophys. Acta* **1973**, *292*, 291. (d) Gupta, R. K.; Redfield, A. G. *Science* **1970**, *169*, 1204.
(12) (a) Kowalsky, A. *Biochemistry* **1965**, *4*, 2382. (b) Oldfield, E.; Allerhand, A. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3531. (c) Timkovich, R.; Cai, M. L.; Dixon, D. W. *Biochem. Biophys. Res. Commun.* **1988**, *150*, 1044. (d) Concar, D. W.; Whitford, D.; Williams, J. P. *Eur. J. Biochem.* **1991**, *199*, 553. (e) Concar, D. W.; Whitford, D.; Pielak, G.; William, R. P. J. *J. Am. Chem. Soc.* **1991**, *113*, 2401.
(13) (a) Dixon, D. W.; Hong, X.; Woehler, S. E. *Biophys. J.* **1989**, *56*, 339. (b) Dixon, D. W.; Hong, X.; Woehler, S. E.; Mauk, A. G.; Sishta, B. P. *J. Am. Chem. Soc.* **1990**, *112*, 1082. (c) Dixon, D. W.; Hong, X. *Adv. Chem. Ser.* **1990**, *226*, 162.
(14) Andrew, S. M.; Thomasson, K. A.; Northrup, S. H. *J. Am. Chem. Soc.* **1993**, *115*, 5516.
(15) Margoliash, E.; Schejter, A. *Adv. Protein Chem.* **1966**, *21*, 113.
(16) Van Leeuwen, J. W. *Biochim. Biophys. Acta* **1983**, *743*, 408.
(17) (a) Chieng, J.; Zhou, J. S.; Kostic, N. M. *Inorg. Chem* **1994**, *33*, 1600. (b) Guillemette, J. G.; Barker, P. D.; Eltis, L. D.; Lo, T. P.; Smith, M.; Brayer, G. D.; Mauk, A. G. *Biochimie* **1994**, *76*, 592.
(18) (a) George, P.; Glauser, S. T.; Schejter, A. *J. Biol. Chem.* **1967**, *242*, 1690. (b) Schejter, A.; Aviram, I. *J. Biol. Chem.* **1970**, *245*, 1552. (c) Schejter, A.; Plotkin, B. *Biochem. J.* **1988**, *255*, 353.(d) Schejter, A; Plotkin, B.; Vig, I. *FEBS Lett.* **1991**, *280*, 199.
(19) Sutin, N.; Yandell, J. K. *J. Biol. Chem.* **1972**, *247*, 6932.
(20) Bechtold, R.; Gardinner, M. B.; Kazmi, A.; Van Hemelrick, B.; Isied, S. S. *J. Phys. Chem.* **1986**, *90*, 3800.

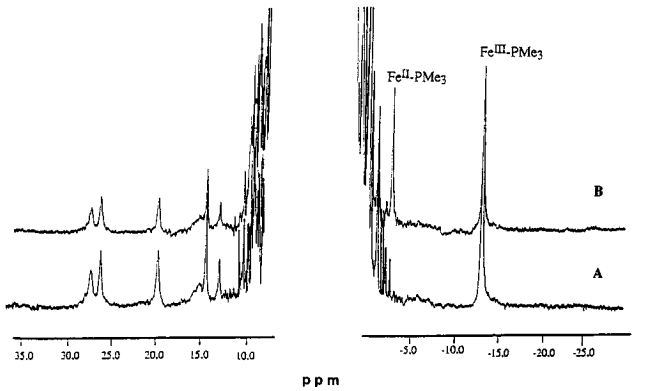

Figure 2. ^{1}H NMR spectra of cytochrome *c* PMe_3: A, ferric state; B, partially reduced state (Fe(III)/Fe(II) = 0.6).

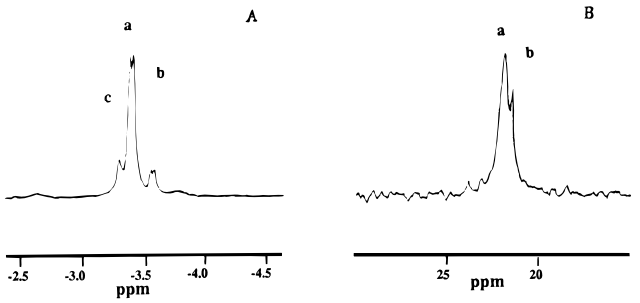

Figure 3. NMR spectra of ferrous cytochrome *c* PMe_3: A, high field portion of the proton spectrum; B, phosphorus spectrum. a and b represent the PMe_3 signals of the major and the minor forms of the phosphine adduct, respectively; c corresponds to the methionine signal of the native cytochrome *c*.

from some time that the thioether sulfur bond of axial methionine is very strong in the reduced state of the iron.[15,18] To make possible the binding of trimethylphosphine to ferrocytochrome *c*, it was first thought that the methionine-80 sulphur atom must be alkylated to displace this ligand from the sixth coordination position in both oxidation states.[18c,d] Thus the binding of trimethylphosphine to the axial position of the heme iron in modified cytochrome *c* by alkylation of methionine (cytochrome *c*−DMC) was first studied by nuclear magnetic resonance spectroscopy.[10] The self-exchange rate constant was 7.5×10^3 M^{-1} s^{-1} at 25 °C in 0.1 M phosphate at pH 7.0. Under the experimental conditions this exchange was slightly slower than the corresponding reaction in unmodified cytochrome *c* ($k = 9.5 \times 10^3$ M^{-1} s^{-1}). However the effect of alkylation of methionyl residues on the electron transfer properties of horse cytochrome *c*[18] must be taken into account because the S-alkylation introduces two more charges in the vicinity of the heme pocket. To avoid this problem, we decided to perform all the experiments without chemical modification of the axial methionine.

Addition of trimethylphosphine (3 equiv) to oxidized cytochrome *c* immediately gave a phosphine cytochrome *c* complex characterized by its ^{1}H NMR spectrum. The methyl proton resonance of PMe_3 ($\delta = -13.6$ ppm) is shifted from the bulk of the protein resonances. Such a spectral feature is characteristic of PMe_3 complexation[8,10,22a] (Figure 2A). The high-field shift is characteristic of a low-spin Fe(III) spin state in the protein, as it was previously reported from NMR studies on ferriporphyrin models.[22b]

The complex of reduced cytochrome *c* was obtained by adding PMe_3 (3 equiv) to oxidized cytochrome *c* followed by sodium dithionite reduction (3 equiv). The high field portion of the ^{1}H NMR spectrum of the reduced form is shown in Figure 3A. The methyl proton resonance of PMe_3 ($\delta = -3.4$ ppm) is shifted upfield from the bulk of the protein resonances. (This signal appears as a doublet, due to ^{1}H−^{31}P coupling.) Such a result, which was previously observed with myoglobin (Fe[II])[8] and hemoglobin (Fe[II]),[22] is due to the shielding effect of the porphyrin ring current. The PMe_3 complex is also characterized by its electronic spectum: $\lambda_{max} = 425$, 527, and 554 nm, in comparison with similar complexes.[22] However, as shown in the ^{1}H NMR spectrum, two other weak signals can be seen together with this intense signal (Figure 3A). One can be easily identified as the methyl group of the ligated methionine in the native ferrocytochrome *c*.[23] The other one is a doublet and is probably due to the phosphine complexation of a minor form of cytochrome *c*. This result is confirmed by the presence of signals at 21.52 and 22.0 ppm in the ^{31}P NMR spectrum of ferrocytochrome *c* PMe_3 (Figure 3B). It is known that cytochrome *c* exists in several pH-dependent forms, in particular two reversible forms were described in the alkaline state.[24] However the transition usually occurs at much higher pH's (9−11) than the pH used here (6.9).

The 300 MHz ^{1}H NMR spectrum of a mixture of reduced and oxidized PMe_3 complexes of cyt *c* is illustrated in Figure 2B. The presence of ferricytochrome *c* has no detectable effect on NMR line width in any of the two proteins. However, slow chemical exchange was evident from the measurement of saturation transfer. Thus cytochrome *c* can be cycled between its two oxidation states by virtue of electron exchange.

Inversion recovery techniques were used to measure the rate constant for electron exchange.[8,10,11,22a] According to the classical theory of magnetization transfer, in the case of moderately rapid reaction, the self-exchange rate constant can be determined by measuring the spin−lattice relaxation time.[11a] The longitudinal relaxation times of PMe_3 on the reduced state were measured in absence ($T_1^{Red} = 370$ ms) and in presence (T_{1a}^{Red}) of oxidized cytochrome *c* PMe_3. Measurements were performed using conventional inversion recovery pulse sequence (see Experimental Section). Knowing these values in the reduced state enabled the life-time τ_{Red} to be estimated, using the equation

$$(T_{1a}^{Red})^{-1} = (T_1^{Red})^{-1} + (\tau_{Red})^{-1}$$

From an integration of NMR peaks and spectrophotometric measurements, the concentration of ferrous and ferricytochrome *c* PMe_3 was known and used to calculate bimolecular self-exchange rates, k_1, using the equation

$$k_1 = k_{-1} = 1/\tau_{Red}[Ox]$$

$$*Fe^{II} + Fe^{II} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} *Fe^{II} + Fe^{III}$$

In phosphate buffer, at pH 6.9 and 23 °C, the bimolecular rate constant for self-exchange for horse heart cytochrome *c* is

(21) (a) Shao, W.; Yao, Y.; Liu, G.; Tang, W. *Inorg. Chem.* **1993**, *32*, 6112. (b) Shao, W.; Sun, H.; Yao, Y.; Tang, W. *Inorg. Chem.* **1995**, *34*, 680.

(22) (a) Simonneaux, G.; Bondon, A.; Brunel, C.; Sodano, P. *J. Am. Chem. Soc.* **1988**, *110*, 7637. (b) Simonneaux, G.; Sodano, P. *Inorg. Chem.* **1988**, *27*, 3956.

(23) Wand, A. J.; Di Stephano, D. L.; Feng, Y.; Roder, H.; Englander, S. W. *Biochemistry* **1989**, *28*, 186.

(24) (a) Brautigan, D. L.; Feinberg, B. A.; Hoffman, B. M.; Margoliash, E.; Peisach, J.; Blumberg, W. E. *J. Biol. Chem.* **1977**, *252*, 574. (b) Barker, P. D.; Mauk, A. G. *J. Am. Chem. Soc.* **1992**, *114*, 3619. (c) Goldkorn, T.; Schejter, A. *FEBS Lett.* **1977**, *75*, 44. (d) Goldkorn, T.; Schejter, A. *FEBS Lett.* **1977**, *82*, 293. (e) Hong, X.; Dixon, D. W. *FEBS Lett.* **1989**, *246*, 105.

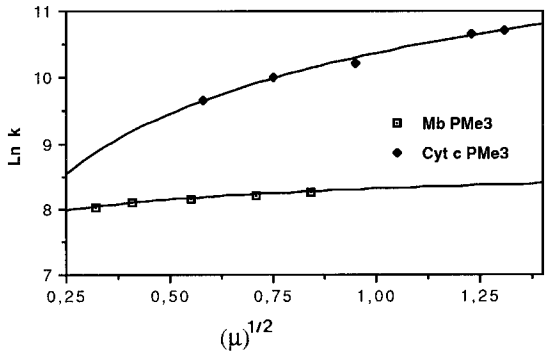

Figure 4. Ionic strength dependence of the electron transfer self-exchange rate constant of cytochrome *c* PMe_3 and myoglobin PMe_3[8a] (pH 6.9, 25 °C, 0.1 M potassium phosphate buffer). The solid lines are the best fit of the data to the van Leeuwen equation.

1.56×10^4 M^{-1} s^{-1} ($\mu = 0.34$ M). As seen from the ionic strength dependence (Figure 4), the rate constants increase with ionic strength. For example, the rate under the previous conditions but with added KCl to 1.7 M was 4.5×10^4 M^{-1} s^{-1}. To follow the ionic strength dependance of the rate of a reaction between two large proteins with a dipole moment, we used the van Leeuwen approach[16] (see Experimental Section). The dipole projections through the heme edge, previously calculated by Dixon et al., are 300 and 275 D for native ferri- and ferrocytochrome *c*, respectively.[13c] We assume a similar situation in the phosphine ligated state. The method also assumes that electron transfer occurs at the partially exposed heme edge. To check the validity of this supposition, the charges of cytochrome *c* were calculated from a fit of the self-exchange data, using the van Leeuwen approach. The solid line in Figure 4 is a best fit to the data for these dipole moments. This gives $Z = 6.2$ and $Z = 7.2$, very close to the values previously reported for native ferro- (6.5) and ferri cytochrome (7.5) *c*.[13c] The value in 0.1 M potassium phosphate buffer without any added KCl ($k = 1.2 \times 10^4$ M^{-1} s^{-1}) was not taken into account in the fit because the presence of excess protonated phosphine decreases the accuracy of the measurement at low ionic strength. An extrapolation to infinite ionic strength of a plot of the ionic strength dependence of the self-exchange rate between cytochrome *c* PMe_3 versus $f(\mu^{1/2})$ yields $k_{inf} = 3.9 \times 10^5$ M^{-1} s^{-1} (Figure 4). The method also allows the determination of an interaction energy, w_r, of 2.06 kcal mol^{-1} [$w_r = -RT \ln(k_1/k_{inf})$] for the two cytochromes in a heme edge-to-heme edge geometry.

To compare myoglobin and cytochrome *c*, a similar approach was carried out with our values previously obtained with horse heart myoglobin PMe_3.[8] Myoglobin has a dipole moment of 301 D in the ferrous state.[17] The dipole moment in the ferric state and k_{inf} were obtained by fitting the experimental data to the van Leeuwen equation. This gives a dipole moment of 320 D for the ferric state (dipole projection through heme edge: 230 D) and a value of $k_{inf} = 7.5 \times 10^3$ M^{-1} s^{-1}.

Discussion

We first comment on the unusual stability of ferrocytochrome *c*−PMe_3. In ferric cytochrome *c*, cyanide and imidazole displace the methionine ligand whereas substitution of this ligand is not observed when the iron is reduced.[25] The hypothesis that the iron−methionine sulfur bond is enhanced by delocalization of the metal t_{2g} electron into the empty 3d orbitals of the sulfur atom in the ferrous state was suggested by Schejter and collaborators to explain this stability.[18d] To make possible the binding of ligands to native ferrocytochrome *c*, we first substituted the methionine by the ligand in the ferric state of the protein, and then reduced the complex to the ferrous state. Addition of cyanide to ferricytochrome *c* followed by dithionite reduction yielded a ferrous transient intermediate ($t_{1/2} < 2$ s) with a spectrum ($\lambda_{max} = 420$, 524, and 552 nm) which is very similar to that obtained from addition of cyanide to ferrocytochrome *c*−DMC.[18] On the other hand, addition of phosphine to ferricytochrome *c* followed by reduction yielded a stable ferrous adduct ($t_{1/2} > 12$ h) characterized by its distinctive electronic spectrum, $\lambda_{max} = 424$, 527, and 554 nm and its ^{1}H NMR spectrum. The latter is very similar to the spectrum obtained after addition of PMe_3 to cytochrome *c*−DMC.[10] The relative stability observed with the phosphine may be related to the large steric effect due to the presence of the bulky phosphine ligand[26] which prevents the complexation of the methionine.[27]

As mentioned in the introduction, the determination of electron rate constants for many biological systems is becoming an increasingly important problem in the current theoritical and experimental investigation. As more experimental values become available for the actual metalloproteins, trends are beginning to appear which supply some insight into the nature of the electron transfer mechanism in these systems. The self-exchange rate of electron transfer for horse-heart cytochrome *c* is fast compared to the rate for myoglobin. There has been recent discussion of these facile electron transfer reactions. Most emphasis has been placed on the nature of the medium separating the metal centers. Less attention has been focused on the possible role of the coordination sphere surrounding the redox center in minimizing the reorganization energy upon a change and thus maximizing the rate of electron transfer.

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.[13] These authors have obtained a value of 0.72 eV for cytochrome *c* and a value of 1.2 eV for cytochrome b_5. We also recently reported a value of 1.6 eV for $MbPMe_3$.[8a] With the use of the Marcus formalism,[2] the cytochrome *c* PMe_3 self-exchange rate constant can be expressed as

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

where S is the steric factor, K_a is the association constant for formation of the precursor state from the two proteins, ν_n is the nuclear frequency factor, κ_{el} is the probability of electron tunneling, and ΔG_r^* 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 Avogadro's number, $r = 33.2$ Å[13a] is the sum of radii of the two proteins, $\delta(r)$ is usually taken as $\beta^{-1} = 1.11$ Å,[13a,28] and with the use of the van Leeuwen formalism, $\exp(-w_r/RT) = \ln(k_1/k_{inf})$. Given these values, we calculate $K_a = 0.287$ M^{-1}. Marcus and Sutin[2] have introduced the steric factor *S* to allow for the fact that there is an angular dependence of electron transfer. A lower limit for the steric factor *S* is equal

(25) (a) Schejter, A.; Aviram, I. *Biochemistry* **1969**, *8*, 149. (b) George, P.; Schejter, A. *J. Biol. Chem.* **1964**, *239*, 1504.

(26) Tolman, C. Z. A. *J. Am. Chem. Soc.* **1970**, *92*, 2956.

(27) Legrand, N. Ph.D. Thesis, University of Rennes 1: Rennes, France, **1994**.

(28) Values of β in the range of 0.9−1.4 A^{-1} have also emerged from studies of long-distance electron transfer reaction in ruthenium-modified myoglobin.[4b] (With $\beta = 1.4$ A^{-1}, for example, the herein reorganization energy is 0.43 eV.) Although β varies with the system , we have chosen $\beta = 0.9$ A^{-1} in the present work since this value is similar to most other estimates of this parameter.[4a,8,13,14]

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 cytochrome *c* is 0.7% heme.[29] Using this assumption and an enhancement factor of 5, Dixon et al.[13a] 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, and $d_0 = 3$ Å and $\beta = 0.9$ Å^{-1} are the standard values.[2,13a]

As previously reported for the cytochrome *c*−cytochrome *c* complex,[13b] we estimate a value of $d = 8.9$ Å for the closest approach in the heme edge-to-heme edge geometry.[30] Therefore, we calculate a value for $\nu_n\kappa_{el}$ of 4.94×10^{10} s^{-1}, and with the above values for K_a, *S* and $\kappa\nu$, the value of ΔG_r^* is 4.3 kcal mol^{-1}. The reorganization energy λ can obtained through application of the following relationships according to the Marcus formalism:

$$\Delta G_r^* = (\lambda/4)[1 + \Delta G^{\circ\prime}/\lambda]^2$$

$$\Delta G^{\circ\prime} = \Delta G^\circ + w_p - w_r$$

where ΔG_r^* is the free energy of activation that is related to λ, to ΔG° 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° 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 cytochrome $c-PMe_3$ is 0.75 eV.

Because, in many hemoproteins, one edge of the heme iron−porphyrin prosthetic group is exposed to the protein surface, as for example cytochrome *c*, the exposed heme edge has been proposed as the site for electron transfer. Actually this situation is implicited in most of the discussion relevant to self-exchange electron transfer. In the absence of a three-dimensional structure for the PMe_3-ligated cytochrome *c*, it is not possible to define rigorously the geometry of the protein, in particular the modification in the heme pocket due to the presence of the phosphine ligand. However such axial ligand interchanges have been recently investigated by replacement of Phe 82 by His in an isocytochrome *c* mutant, resulting in bis-His ligation of the heme,[31] and, more thorougly, by ^{1}H NMR studies of the pyridine and imidazole complexes of cytochrome *c*.[21] In the latter case, some conformational changes are apparent on the distal side of the heme pocket, in particular Phe 82 and Ile 81 seem to move away from the neighbor region of the axial ligand on the basis of NOE data. A schematic diagram, showing the conformational differences of heme pockets between cytochrome *c* and Im−cytochrome *c* was also proposed.[21b] Precisely how this different heme pocket due to the bulky phosphine complexation might transfer electrons cannot be determined on the basis of our current studies. Moreover, the changes in the position of the amino acids on the distal side, due to the presence of the phosphine may not be the sole factor of decreasing the rate at infinite ionic strength. For exemple, it has been recently demonstrated that Phe 82 does not contribute directly to electron transfer, when the thermodynamic driving force of the reaction is weak (less than 100 mV) or zero as it is the case for self-exchange reactions.[12e] The flexibility of the iron coordination sphere, due to the disruption of the metal−sulfur bond, may also be another factor. This situation should increase the reorganization energy because the bond lengths of the axial ligands may be different, as we previously reported on iron porphyrin models.[32] Accordingly, the reorganization energy is higher in cytochrome $c-PMe_3$ (0.75 eV) than that in native cytochrome *c* (0.72 eV).[13b] However, this difference is weak[33,34] and since it has been reported from X-ray structure determinations that there are no significant bond length differences between the heme iron and the ligand atoms between the oxidized and reduced native cytochrome *c*,[33] a definitive conclusion is not possible with the phosphine adduct.

It is also of interest to determine the extent to which the coordination environment of hemoproteins is dictated by the nature of the heme ring (heme *a* or *c*) and to what extent this may influence the rate of self-exchange electron transfer. Comparison of the rates for cytochrome *c* PMe_3 with similar hemoproteins, such as myoglobin PMe_3 permits definition of this factor in greater detail. In a thorough study on the myoglobin(III)/myoglobin(II)−PMe_3 system, we have been able to provide an experimental determination of the self-exchange electron transfer rate constant for the first time.[8a] At infinite ionic strength, the rate constant is 7.5×10^3 M^{-1} s^{-1}. As myoglobin has more heme exposed to solvent than does cytochrome *c*,[29] the main question is, Why does cytochrome *c* exchange electron more rapidly? To illustrate the foregoing point, Figure 1 shows the heme pocket of four hemoproteins cytochrome *c*, cytochrome $c-PMe_3$, cytochrome b_5, and myoglobin−PMe_3 together with the values of the rate constants at infinite ionic strength. There is an increase in the rate constant with an increase in the number of covalent links between the axial ligands and the heme ring. The gap is between cytochromes *c* and b_5 on one hand, and myoglobin on the other. This behavior is not unique to self-exchange systems and has been observed for intramolecular electron transfer reactions in different hemoproteins labeled with ruthenium complexes.[6,7] This situation may suggest a role for the flexibility of the heme coordination sphere, in addition to the usual factors[2] in the modulation of biological electron transfer kinetics. However, much work remains to establish the contribution of these factors, in molecular terms, to the the electron transfer rates.

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(29) Stellwagen, E. *Nature* **1978**, *275*, 73.
(30) As examples, the reorganization energies are 1.02, 0.88, and 0.65 eV with $d = 6$, 7.5, and 10 Å, respectively.
(31) Hawkins, B. K.; Hilgen-Willis, S.; Pielak, G. J.; Dawson, J. H. *J. Am. Chem. Soc.* **1994**, *116*, 3111.
(32) (a) Sodano, P.; Simonneaux, G.; Toupet, L. *J. Chem. Soc., Dalton Trans.* **1988**, 2615. (b) Toupet, L.; Legrand, N.; Bondon, A.; Simonneaux, G. *Acta Crystallogr.* **1994**, *C50*, 1014.
(33) (a) Takano, T.; Dickerson, R. E. *J. Mol. Biol.* **1981**, *153*, 79. (b) Takano, T.; Dickerson, R. E. *J. Mol. Biol.* **1981**, *153*, 95. (c) Berghuis, A. M.; Brayer, G. D. *J. Mol. Biol.* **1992**, *223*, 959.
(34) Gao, Y.; Boyd, J.; Pielak, G. J.; Williams, R. J. P. *Biochemistry* **1991**, *30*, 1928.