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Mechanism of Electron Transfer in Hemoglobin: Evidence of Both Homosubunit and Heterosubunit Intermolecular Electron Transfers

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Abstract: An experimental investigation of the mechanism of electron transfer in trimethylphosphine complexed hemoglobin has been performed. Using saturation transfer experiments with native and [Fe,Mn] hybrid Hb we show that both intra- and interchain electron transfers occur. Calculations based on ^1H NMR spectra and using the inversion-recovery method determined the rate constants to be k_{11} = 3200 M^{-1} s^{-1} (α chains) and k_{22} = 2090 M^{-1} s^{-1} (β chains) for self-exchange transfers, k_{12} = 1020 M^{-1} s^{-1} ([α(II) PMe_3] + [β(III) PMe_3]) and k_{21} = 430 M^{-1} s^{-1} ([α(III) PMe_3] + [β(II) PMe_3] for heterosubunit transfers. The lifetime plotted versus the inverse of ferric hemoglobin concentration shows a good linear relationship, according to a bimolecular reaction without any significant intramolecular contribution. Application of the Marcus relationship to cross reactions yields k_{12} = 3830 M^{-1} s^{-1} and k_{21} = 1730 M^{-1} s^{-1} values which are in good agreement with the k_{12} and k_{21} NMR values indicating that all these electron transfers represent reactions by a single pathway with no evidence of configurationally-limited behavior.

Introduction

The study of electron-transfer self-exchange kinetics has played a major role in the understanding of reaction mechanism governing electron transfer between proteins.[1] The rate of electron transfer between metalloproteins is a function of several factors including the reorganization energy, the solvation, the distance between the donor and acceptor centers, and the nature of the intervening medium. These experiments afford a valuable opportunity for testing theoretical models, but, despite their biological importance, there is little systematic experimental data on rates of bimolecular electron transfer with multiredox center hemoproteins.[2] Most of the studies with these particular systems have been focused on intramolecular[3] or interprotein complex[4] electron transfers.

Myoglobin and hemoglobin are particularly well suited for detailed electron-transfer mechanistic investigation, because both proteins are well characterized, including high-resolution crystal structures.[5] Furthermore, the availability of a convenient method for the preparation of mixed-metal hybrid hemoglobins now permits a new approach to the study of heterosubunit interaction in hemoglobin.[6] At present, experiments that have provided useful information about long-range electron transfer rates have involved, for example, electronically excited zinc-substituted hemoglobin[3a,4] and ruthenium modified myoglobin.[3b,c] For many years the question of the electron self-exchange rate has been of interest but not experimentally accessible because no ligand would strongly[7,8] bind to the iron center in both oxidation states. We have recently shown that electron self-exchange between myoglobins can be studied by trimethylphosphine complexation to heme iron.[9a] A key advantage of this approach is that it permits us to determine the reorganization energy from experimental self-exchange rates because suitable reduced and oxidized states have been now available.[9] We now report the first example of direct[10] measurements of intermolecular electron transfer rates, including the self–exchange rates, between hemes in hemoglobins which indicates that the mechanism involves both heterosubunit and homosubunit exchanges. Analysis of

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(7) Besides the phosphine mentioned here, nitrogen bases also bind to hemoglobin in both oxidation states. However the binding of these ligands varies enormously between the ferrous and the ferric oxidation states. The structural perturbations observed upon binding of imidazole to ferric horse hemoglobin, for example, are so important that the crystals do not tolerate full ligand saturation: Bell, J. A.; Korszun, Z. R.; Moffat, K. *J. Mol. Biol.* **1981**, *147*, 325.

(8) The autoreduction of ferric porphyrins by PMe_3 has been previously reported: La Mar, G.; Del Gaudio, J. *Bioinorg. Chem.* **1977**, *2*, 207. This difficulty has been circumvented by using the perchlorate derivatives of ferric porphyrins (Simonneaux, G.; Sodano, P. *Inorg. Chem.* **1988**, *27*, 3956). We have also examined the stability of the $metHbPMe_3$ under our experimental conditions. It is found that less than 5% of metHb (no spectral change) was reduced in 4 h under these conditions. However, we noticed that a large excess of PMe_3 induced partial reduction of metHb.

(9) (a) Brunel, C.; Bondon, A; Simonneaux, G. *Biochim. Biophys. Acta* **1992**, *1101*, 73. (b) Simonneaux, G.; Bondon, A.; Brunel, C.; Sodano, P. *J. Am. Chem. Soc.* **1988**, *110*, 7637. (c) Simonneaux, G.; Bondon, A.; Sodano, P. *Inorg. Chem.* **1987**, *26*, 3636. (d) Bondon, A.; Simonneaux, G. *Biophys. Chem.* **1990**, *37*, 407.

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the kinetic results in term of the Marcus theory permits us to make comparison between experimental and calculated cross-reaction rates. The good level of agreement supports the applicability of the Marcus equation to cross reactions involving intermolecular heterosubunit transfers.

Experimental Section

Reagents. Adult human hemoglobin was prepared according to standard method as previously described.[9b,c] The α and β subunits of human hemoglobin were separated and purified as described by Geraci et al.[11] The [Mn,Fe][12] hybrid hemoglobins were prepared following the procedure of Blough and Hoffman[6] with minor modification.[9d] Trimethylphosphine complexation to native or hybrid hemoglobins was carried out by methods which have already been published.[9] For the experiments utilizing a mixture of metHb/Hb, the appropriate equivalent of sodium dithionite was added to metHb in D_2O. Sodium dithionite (Aldrich) was used without further purification. Samples were run in 0.1 M phosphate buffer (pH 7.1) with native hemoglobin. An alkaline pH (8.2) was used for the preparation of samples containing hybrid hemoglobins.

Saturation Transfer Experiments. The experiments were conducted using the truncated driven NOE pulse sequence.[13] To specifically saturate a given trimethylphosphine resonance, a selective decoupler pulse was used during 250 ms. Two spectra were recorded, the first with the saturation pulse on the resonance, the second with the saturation pulse off-set by 600 Hz, thus providing a reference for the difference spectrum. Scans (768) were collected per spectrum and the recycle time was 1 s.

Kinetic Measurements. For measurements of the lifetime, 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 pulse. Each measurement had a series of 25 different τ values. Data were taken in blocks of 64 scans. The details of the method are described elsewhere.[9a] The ratio of oxidized to reduced heme in each subunit was determined from the integrated areas of the two forms of the phosphine bound to α and β chains subunits and then adjusted in order to equalize the amount of α and β chains. The data were analyzed by using the method previously reported by Gupta and Redfield.[14] For the reduced state methyl resonance of PMe_3, the nonselective T_1's were determined as 460 ± 10 ms for the α subunit and as 320 ± 10 ms for the β subunit in the absence of methemoglobin. A selective 180° pulse, inverting only PMe_3 peaks at −3.40 ppm (α subunit) and −3.23 ppm (β subunit) was also used. In this case, the T_1's for the reduced-state methyl resonances of PMe_3 were determined as 80 (α subunit) and 73 ms (β subunit). As expected, this gives shorter T_1 values than the method in which all the peaks are inverted, due to the contribution of nuclear Overhauser effect. The rates constants were calculated from the nonselective method, as we have previously reported.[9a] The spin−lattice relaxation times T_1's (nonselective pulse) were close to 3 ms in the completely oxidized states for the phosphine protons both in the α and β subunits. The lifetimes reported in Table 1 were calculated from the measured values of T_1's in the Fe(II) protein. This method is applicable only when the lifetime in the oxidized state is long compared to the spin−lattice relaxation time in this state.[14] The spin−lattice relaxation T_1 (nonselective pulse) was 460 ms in the completely reduced state of [αFe (PMe_3), βMn] hybrid hemoglobin for the phosphine protons.

Proton NMR spectra were recorded on a Bruker AC 300 P spectrometer in a temperature-regulated probe (25 °C). Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through the residual water resonance.

(11) Geraci, G.; Parkhurst, L. J.; Gibson, Q. H. *J. Biol. Chem.* **1969**, *244*, 4664.

(12) Abbreviations: Hb, hemoglobin; metHb, ferric hemoglobin; [Mn,-Fe], hemoglobin derivative in which the two chains of a single type, α or β, are substituted with Mn protoporphyrin IX. [α(II) PMe_3] indicates PMe_3 ligation of α subunits in the ferrous state. When a particular hybrid is discussed, it is so indicated, e.g., [αFe(III) PMe_3, βMn]. The α/β ratio has been determined from the ratio of the integrated areas of the α and β PMe_3 resonances in the NMR spectra.

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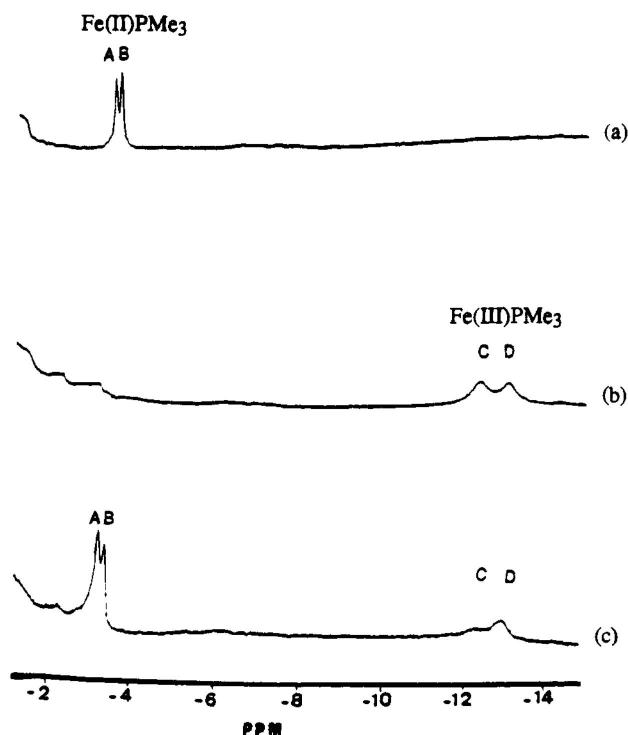


Figure 1. The effect of $metHbPMe_3$ addition on the relative magnitude of the intensity of the PMe_3 methyl resonances of $HbPMe_3$ (high field portion of the spectra). Assignment of the methyl group resonances—Fe(II) PMe_3, A (β chains) and B (α chains) and Fe(III) PMe_3, C (β chains) and D (α chains): (a) $HbPMe_3$, (b) $metHbPMe_3$, and (c) mixture of human hemoglobin $HbPMe_3$/$metHbPMe_3$ in the ratio 65/35 in D_2O at 23 °C (pH 6.9).

Results

Equilibrium Study and Redox Potential. The ^1H NMR spectrum recorded after addition of reduced (Figure 1a) and oxidized (Figure 1b) trimethylphosphine hemoglobin shows four resonances: two resonances from Fe(II) subunits [A (β chains) and B (α chains)][9b] and two resonances from Fe(III) subunits [C (β chains) and D (α chains)][9c] which are clearly seen in the upfield region (Figure 1c). The α/β ratios for the ferric and ferrous states are 1.6 and 0.74, respectively, when 60% of the subunits are in the reduced state (Table 1). Because the ratio between α chains and β chains are different from unity in both redox states, we may assume that electron-exchange occurs between the different subunits. On this basis, the oxidation−reduction potentials of α/β chains are different, the electron affinity of the β chains being higher than that of the α chains. Such a difference in the redox potential has been previously reported with unligated native hemoglobin (see discussion).[15,16]

In order to confirm this heterosubunit electron transfer, a different experiment was set up, using [Mn,Fe] hybrid hemoglobins. As previously reported by Hoffman and Blough, hybrid hemoglobins which are substituted with manganese protoporphyrin IX do not bind CO ligand.[6a] We also reported trimethylphosphine complexation of such hybrids in the iron reduced states leaving the manganese unligated: [αFe(II) PMe_3, βMn] and [αMn, βFe(II) PMe_3]Hb.[9d] This work was extended to the complexation of trimethylphosphine to [Fe(III),Mn] hybrids. The ^1H NMR spectra of two hybrids in a different oxidation state are reported in Figure 2a ([αFe(III) PMe_3, βMn]) and Figure 2b ([αMn, βFe(II) PMe_3]), respectively. The spectrum of a

(15) Banerjee, R.; Cassoly, R. *J. Mol. Biol.* **1969**, *42*, 337.

(16) Brunori, M.; Alfsen, A.; Saggese, U.; Antonini, E.; Wyman, J. *J. Biol. Chem.* **1968**, *243*, 2950.

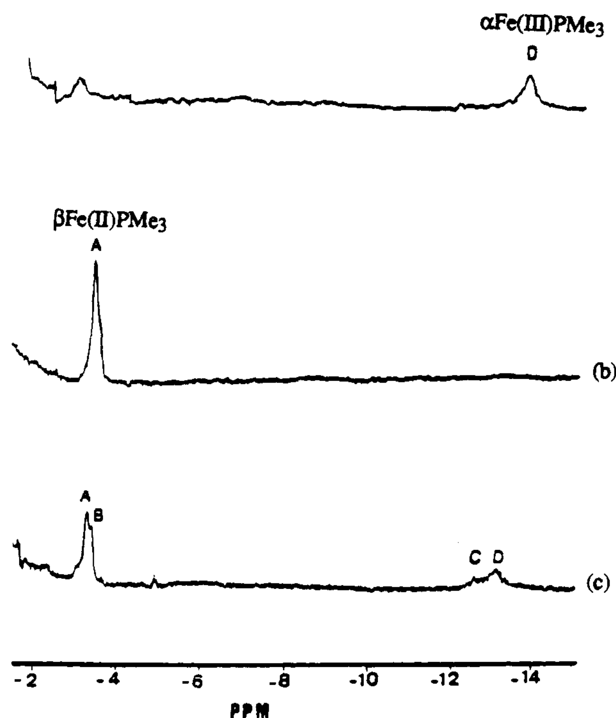


Figure 2. High field ^{1}H NMR spectra showing the interchain electron transfer resulting from addition of [αFe(III) (PMe$_3$), βMn] hybrid hemoglobin (a) to αMn, βFe(II) (PMe$_3$) (b). (c) Equimolar mixture of [αFe(III) (PMe$_3$), βMn] hybrid hemoglobin and [αMn, βFe(II) (PMe$_3$)] (a + b).

Table 1. Lifetimes of Reduced α and β Subunits of Human Hemoglobin at Different Concentrations[a]

α(II) (mM)	α(III) (mM)	β(II) (mM)	β(III) (mM)	τ_α (s)	τ_β (s)
0.97	0.90	1.31	0.56	0.31	0.67
1.21	1.13	1.64	0.70	0.26	0.50
1.56	1.56	2.18	0.94	0.18	0.35
1.85	1.87	2.61	1.11	0.15	0.32

[a] The percent of reduction is maintained constant ([Fe(II)] = 60%). The α/β ratios for ferric and ferrous states are 1.6 and 0.74, respectively.

mixture of these hybrids (Figure 2c) shows four resonances which are very similar to those observed with native hemoglobin (see Figure 1c) in agreement with an heterosubunit electron transfer. Determination of the constant of the oxidation–reduction equilibrium from different concentrations yields to a value of $K_{12} = 2.2 \pm 0.2$ (Table 1).

Saturation Transfer Experiments. Concordant with previous observation in a mixture of oxidized and reduced trimethylphosphine myoglobin,[9a] the presence of ferrihemoglobin (metHbPMe$_3$) in samples of ferrohemoglobin (HbPMe$_3$) had no detectable effect on NMR line widths in any of the proteins. However, slow chemical exchange between ferro- and ferrihemoglobin was also evident from the measurement of saturation transfer. Preirradiation at the frequency of the phosphine methyl resonance of one chain of ferrihemoglobin, at a power level sufficient to abolish the resonance in the spectrum, caused a decrease of the intensity of the corresponding resonance (PMe$_3$) of the same chain of ferrohemoglobin (Figure 3a,b). Because hemoglobin can be cycled between its two oxidation states by virtue of electron exchange with a second, different, redox species present in the same solution, it is important to obtain further information on the origin of the electron transfer.

Electron transfer between ferriheme and ferroheme in native hemoglobin might in principle occur between α/β and/or α/α (β/β) subunits. In order to check for possible homosubunit electron transfers, we set saturation transfer experiments on

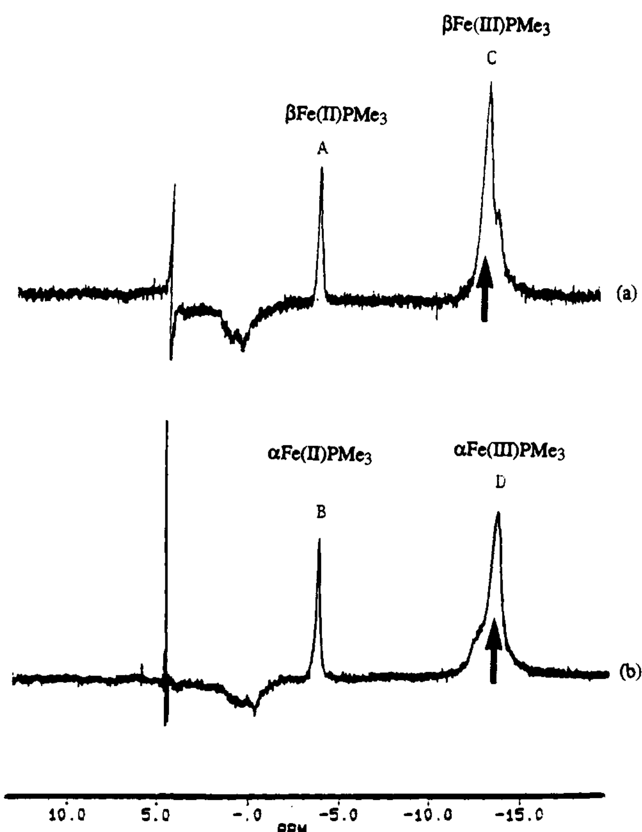


Figure 3. ^{1}H NMR difference spectra (300-MHz) showing saturation transfer from the phosphine methyl groups of metHbPMe$_3$ to the corresponding groups of HbPMe$_3$ for β chains (a) and α chains (b), respectively.

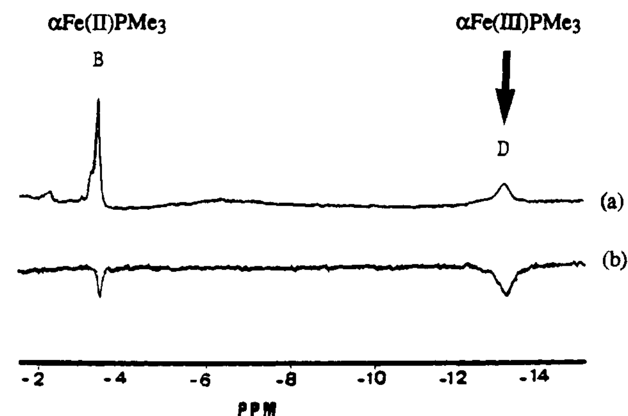


Figure 4. ^{1}H NMR spectra showing saturation transfer from the phosphine methyl groups of [αFe(III) (PMe$_3$), βMn] hybrid hemoglobin to the corresponding groups of αFe(II) (PMe$_3$), βMn] hybrid hemoglobin: (a) reference spectrum and (b) difference spectrum after irradiation.

mixed-metal [αFePMe$_3$,βMn] hybrid hemoglobins. Slow homosubunit chemical exchange between ferro and ferri subunits was also evident from saturation transfer in the two α (PMe$_3$) hybrids (Figure 4), in agreement with homosubunit electron transfer. Similar results have been obtained with the β (PMe$_3$) hybrids (not shown).

Relaxation Measurements. The mechanism of electron transfer might be intramolecular or (and) intermolecular. In order to obtain information to this point, the dependence of the lifetime ($1/\tau_{red}$) of the PMe$_3$ resonances of both chains, as a function of the concentration of exchanging species, was first investigated (Table 1). At constant ionic strength (0.1 M phosphate buffer) and near neutral pH, the lifetime of the ferrous

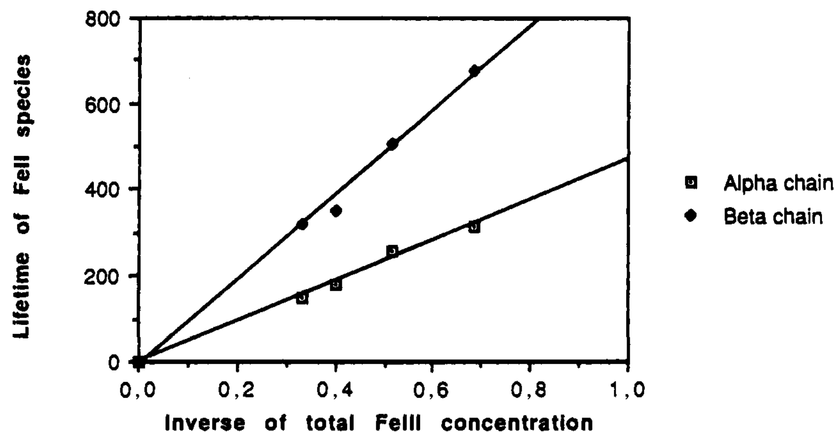
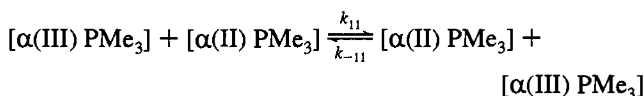
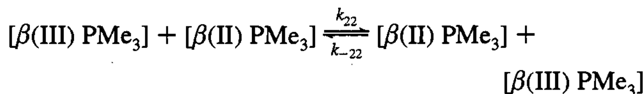


Figure 5. The lifetime dependance of reduced α and β subunits of human hemoglobin as a function of the ferrihemoglobin concentration at constant Fe(II)/Fe(III) ratio (Fe(II) = 60%).

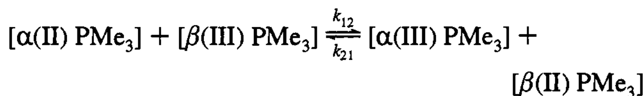
state (rate of exchange process) shows first order decrease with the inverse of the ferrihemoglobin concentration (Figure 5), with zero intercept. Therefore intramolecular exchange has little (if any) influence on the rate of the reaction. Besides establishing an exchange of protein molecules between different oxidation states, the technique can also be used to measure the kinetics of the redox reactions. However, because each subunit can be cycled between its two oxidation states by virtue of electron exchange with two different redox subunits, the exchange process is therefore



$$[\alpha(\mathrm{III})\,\mathrm{PMe_3}] + [\alpha(\mathrm{II})\,\mathrm{PMe_3}] \underset{k_{-11}}{\overset{k_{11}}{\rightleftharpoons}} [\alpha(\mathrm{II})\,\mathrm{PMe_3}] + [\alpha(\mathrm{III})\,\mathrm{PMe_3}]$$



$$[\beta(\mathrm{III})\,\mathrm{PMe_3}] + [\beta(\mathrm{II})\,\mathrm{PMe_3}] \underset{k_{-22}}{\overset{k_{22}}{\rightleftharpoons}} [\beta(\mathrm{II})\,\mathrm{PMe_3}] + [\beta(\mathrm{III})\,\mathrm{PMe_3}]$$



$$[\alpha(\mathrm{II})\,\mathrm{PMe_3}] + [\beta(\mathrm{III})\,\mathrm{PMe_3}] \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} [\alpha(\mathrm{III})\,\mathrm{PMe_3}] + [\beta(\mathrm{II})\,\mathrm{PMe_3}]$$

where α(II) and α(III) are the α chains in, respectively, the ferrous and the ferric state; k_{11} and k_{12} are, respectively, the self-exchange constant for the homosubunit reaction and the cross-exchange constant for the heterosubunit reaction. The lifetimes of the two reduced species are described by the following equations

$$\tau_{\alpha(\mathrm{II})} = \alpha(\mathrm{II})/k_{11}[\alpha(\mathrm{II})][\alpha(\mathrm{III})] + k_{12}[\alpha(\mathrm{II})][\beta(\mathrm{III})] \quad (1)$$

$$\tau_{\beta(\mathrm{II})} = \beta(\mathrm{II})/k_{22}[\beta(\mathrm{II})][\beta(\mathrm{III})] + k_{21}[\beta(\mathrm{II})][\alpha(\mathrm{III})] \quad (2)$$

k_{11}, k_{22}, k_{12}, and k_{21} values should be obtained through application of (1) and (2) relationships using different concentrations. However the values could only be obtained using a large difference for the percentages of reduction. This was not possible because (i) the ratio between the two subunits is controlled by the weak difference in the redox potential and (ii) the accuracy of the T_1 measurements requires that the Fe(II)/Fe(III) ratio is maintained in a relatively narrow range. In order to solve this problem, the self-exchange constant between the α chains (k_{11}) was calculated from the determination of the lifetime of reduced α chains, using an experiment on mixed-metal hybrid hemoglobin. In 0.1 M potassium phosphate buffer (pH 8.2) and 23 °C, the bimolecular rate constant for self-exchange for α chains is 3.2×10^3 $\mathrm{M^{-1}\,s^{-1}}$. We assume that this value is maintained with native hemoglobin,[17] and we use the following relationships (the k_{12}/k_{21} ratio can be estimated as 2.2, see Table 1)

$$K_{12} = [\alpha(\mathrm{III})][\beta(\mathrm{II})]/[\alpha(\mathrm{II})][\beta(\mathrm{III})] = k_{12}/k_{21} \quad (3)$$

$$k_{12}[\alpha(\mathrm{II})][\beta(\mathrm{III})] = k_{21}[\alpha(\mathrm{III})][\beta(\mathrm{II})] \quad (4)$$

Therefore, the determination of the lifetimes, summarized in Table 1, allows the calculation of all the constants: $k_{11} = 3200$, $k_{22} = 2090$, $k_{12} = 1020$, and $k_{21} = 430$ $\mathrm{M^{-1}\,s^{-1}}$. Thus the contribution of the interchain process in the overall process can be estimated.

Discussion

Mechanism. Since the report of the first direct evidence for electron exchange between ferrous and ferri cytochrome *c*,[14,18,19] the study of electron self-exchange reactions in hemoproteins has been largely developed.[20] However most of the work focused on one redox metal site and self-exchange reactions with multisites are still rare.[2] In studying the mechanism of self-exchange electron transfer in hemoglobin, there are at least two major pathways to consider. First, the electron transfer may be intramolecular. An original intramolecular approach has used Zn-substituted hemoglobins in which the distance and orientation of electron donor and acceptor are fixed.[3a,4] The reported first-order rate of electron transfer is 350 $\mathrm{s^{-1}}$. However it should be noted that the thermodynamic driving force is quite important in these systems.[21] The values of the lifetime,

(17) The [αMn, βFe(III) (PMe₃)] system was not investigated because the Fe(II)/Fe(III) β subunit ratio did not remain constant over the prolonged data acquisition times required to measure rate constants. This is due to a small instability of β chains in the ferric state. However, as we previously observed with trimethylphosphine myoglobin,[9] preliminary experiments on the influence of the pH (6–8) and the ionic strength (0.1–0.8 M) to the rates show also a very weak contribution of these two factors.

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determined in the current study, were plotted versus the inverse of ferric hemoglobin concentration (Figure 5). We note that a good linear relationship is obtained with zero intercept, according to bimolecular reactions without any significant intramolecular contribution.

Once the intramolecular pathway has been ruled out, we must consider two different intermolecular pathways: the homosubunit and the herosubunit pathways. The saturation transfer experiments support the involvement of both the homosubunit and herosubunit mechanisms (Figures 3 and 4). It is therefore of interest to calculate each contribution. The self-exchange rate constant obtained for the α chains is 3200 M^{-1} s^{-1} which is weakly higher than that for the β chains (2090 M^{-1} s^{-1}). For the current study, the direct determination of the lifetime makes possible the measurements of cross-reaction kinetics for both α and β subunits. We note that both values are smaller than those obtained for self-exchange reactions, though the origin of this difference is not apparent at present. In early work, it was shown that the respective reactivities of the α and β subunits are quite similar in the cytochrome b_5—hemoglobin system.[22] Though there are some conflicting results in which it was stated that the β subunit is more rapidly reduced than the α subunit,[23] further studies assume largely that the two subunits are reduced with the same rate.[24,25] Our kinetic results for the self-exchange electron transfers of hemoglobin are also consistent with a weak difference between the subunits although the two systems, i.e., the b_5—hemoglobin complex and the self-exchange transfer, are quite different. The former system is directly related to a true complex with complementary charges near the active site, while in the latter, identical charges surrounding the heme pocket will disfavor the interaction and the electron transfer.

Redox Potential. It has been known since the work of Banerjee[15] and Brunori[16] that the oxidation—reduction potentials of α and β chains are different, indicating preferential oxidation of α chains. This chain heterogeneity may be the cause of decreased cooperativity of the oxidation—reduction of hemoglobin.[26] It is also of interest to point out that the difference in chain concentration in both trimethylphosphine iron redox states corresponds to a difference in oxidation—reduction potentials which is equal to 21 mV. The oxidation—reduction equilibrium of the isolated α and β chains[15] and of haptoglobin-bound hemoglobin[16] showed a difference in the redox potentials of 61 and 56 mV, respectively. Thus, complexation of trimethylphosphine gives also a difference which is in the same sense (β chains more easily reduced) but with a smaller value.

Self-Exchange and **Cross Reactions of Hemoglobin.** The simplified Marcus cross relations (eqs 5 and 6) have been applied to a number of electron-transfer reactions in hemoproteins.[1a]

$$k_{12} = (k_{11}k_{22}K_{12}f_{12})^{1/2} \quad (5)$$

$$\log f_{12} = (\log K_{12})^2/(4 \log (k_{11}k_{22}/Z^2)) \quad (6)$$

In these equations, k_{12} and K_{12} are the rate and equilibrium constants for the cross reaction, respectively, k_{11} and k_{22} are the self-exchange rate constants, and Z is the collision frequency of two uncharged particles in solution (taken as 10^{11} M^{-1} s^{-1}).

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The reported self-exchange rate constant of hemoglobin, calculated from the Marcus theory with various inorganic redox couples, varies from 1.9×10^{-4} to 2.8×10^{-3} M^{-1} s^{-1}.[27] A similar behavior was previously reported with myoglobin,[27] though a higher value of 1 M^{-1} s^{-1} at 25 °C has been reported for the rate constant of the self-exchange reaction of the native metMb/deoxyMb system.[28] Besides this large discrepancy between these theoretical results, no difference between the two subunits was taken into account with hemoglobin. Our experimental result shows a weak difference between the two subunits and values much larger than those reported earlier. As suggested with myoglobin,[29] the geometry change upon reduction of native methemoglobin, which is accompanied by a water dissociation, may explain this difference. Trimethylphosphine complexation in both redox states is expected to give a much lower reorganizational barrier.

For the current study, the experimental availability of the rate constants for self-exchange transfers and cross chain reactions make it possible to test the veracity of the Marcus and Sutin approach.[1a] Utilizing the data obtained from our kinetics, we have calculated[30] the value of the cross reaction rate constants, $k_{12} = 3830$ M^{-1} s^{-1} and $k_{21} = 1730$ M^{-1} s^{-1}. Thus, from a comparison with experimental values ($k_{12} = 1020$ M^{-1} s^{-1} and $k_{21} = 430$ M^{-1} s^{-1}), it is apparent that the application of the Marcus relationship to cross reactions yields k_{12} and k_{21} values which are in good agreement with the NMR k_{12} and k_{21} values. Some of the assumptions and limitations inherent in the above equations have previously been reviewed.[31,32] It was found for reactions between inorganic complexes that reasonable exchange-rate estimates can be obtained with eqs 5 and 6 provided that the driving force for the cross reaction is not too large.[31] In general, in systems in which the best agreement between theory and experiment has been found, the calculated and observed rate constants agree within a factor of ten.[31b] The same sort of analysis has also been applied to the electron transfer reactions in blue copper proteins and hemoproteins,[1a,33,34] but the agreement was sometimes less satisfactory.[35,36] However, the agreement of the observed and calculated exchange is fairly good in the case of cytochrome *c*, whose heme group is located at the surface of the protein.[37] Since the heme groups of hemoglobin are also located at the surface of the globin, a similar behavior may be expected, if it is assumed that the electron transfer reactions of hemoglobin proceed through the exposed heme edge. In addition, the similarity in the heme exposure of the two subunits is striking: Hb_α 14% and Hb_β 20%.[37] As mentioned above, there is evidence that the fit between theory and experiment worsens as the driven force of the reaction

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(35) A comparison of observed and calculated rate constants for protein—protein reactions has been previously reported: see ref 1a, p 305. For a recent analysis, see, for example: ref 34.

increases.[31] By contrast, complexation of trimethylphosphine to Hb gives a difference in oxidation–reduction potentials which is small. Finally, electrostatic interactions are obviously important for self-exchange transfers in proteins,[38] and, in particular, deviations between the observed and the calculated rate constants become larger when the reactants are oppositely charged.[1a,31] However, we previously reported that the rate of self-exchange electron transfer in trimethylphosphine myoglobin was weakly dependent on ionic strength.[9a] A similar situation may occur with the hemoglobin system, but further quantitative developments will be necessary to assess this point. These three factors (similar heme exposure, low driving force, and weak charge effect) may also account for the good agreement between the observed and calculated constants. Thus we can conclude that all these electron transfers represent reactions by a single pathway with no evidence of configurationally-limited behavior.

(36) Some electron transfer reactions are accompanied by a spin change and on this basis should proceed slowly.[1a] This may lead to an additional source for apparent difference between calculated and observed constants in determining electron transfer rates. But in the trimethylphosphine Hb system, this is not the case because both redox states are low spin.

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Conclusion

Our results show that hemoglobin is a good system to study the kinetic aspects of multisite components in electron transfer reactions. We have measured the self-exchange rates and the intermolecular cross-chain rates of electron transfer in hemoglobin. These rates are not accessible to measurements using other methods. The previously noted discrepancies between the calculated and the experimental values are suggested to arise from a geometry change upon reduction in native hemoglobin. Control of the conformational rigidity of the heme pocket is now possible because trimethylphosphine will bind the iron center in both oxidation states. We suggest that most, if not all, heme oxygen carrier systems involve similar behavior.

The bimolecular mechanism we have discovered may have important implication in regard to the binding and electron transfer steps required for the reduction of hemoglobin by cytochrome b_5. In this context the description of the intramolecular electron transfer step previously reported[24,25] at low protein concentration is important. We hope to shed some light on this process by further Hb–b_5 studies with our system.