Mechanism of Electron Transfer in Heme Proteins and Models: The NMR Approach

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1. Introduction

Long-range electron transfer is an essential component of biological systems, playing an important role in respiration and photosynthesis.[1−6] Much current research is centered around probing what factors control the rates of nonadiabatic electron-transfer reaction, including the driving force, reorganization energy, separation distance, and the nature of the medium separating the electron donor and the acceptor of the system.[1,7] The availability of high-resolution NMR or X-ray crystallographic structures for a number of heme proteins has encouraged much work on the electron-transfer properties of these metalloproteins.[8] The crystal structure of a complex between electron-transfer partners, cytochrome *c* peroxidase and cytochrome *c*, reveals a possible electron-transfer pathway unlike any previously proposed for this extensively studied redox pair.[9] Thus, the role of the protein in controlling the rate of electron transfer is still the subject of intense study, such as the extent of heme exposure[10] and orientation,[11] the nature of amino acids on the surface of the proteins,[12,13] ionization[14]or esterification[15] of the heme propionates, and the overall dipole moment.[16] Various approaches have been used to carry out kinetic measurements of long-range electron transfer. These include the following:

(1) Zn-substituted cytochromes to measure the rate of intracomplex electron transfer between cytochrome *c* and a cytochrome *c* peroxidase complex[17] and between nonphysiological complexes (cytochrome *c* / plastocyanin[18] and cytochrome *c*/cytochrome b_5).[19] NMR studies with Zn cytochrome *c* in aqueous solution have demonstrated that the conformation of the protein is unchanged by incorporation of Zn^{2+} in place of Fe^{2+}.[20,21] Long-range electron transfer within mixed-metal hemoglobin hybrids has also been reported, using flash photoproduction of the long-lived triplet state by Hoffman and co-workers.[22] Electron-transfer reactions of hemoglobin with small molecules have been studied by using photoactive zinc-substituted heme.[23]

(2) The use of Ru-modified horse heart cytochrome *c* by coordination of pentaammineruthenium to specific protein sites.[24−26] The first report of such systems appeared in 1982.[27] Such a system was developed mainly by Gray and co-workers, who determined the rates of cytochrome *c* intramolecular electron-transfer reactions under significantly different driving forces.[28]

In the last two examples, the rate constant values for the electron-transfer processes were determined by monitoring transient absorption spectra observed in laser flash photolysis experiments. There are many

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Gérard Simonneaux was born in 1950 and educated at the University of Rennes with G. Jaouen and R. Dabard. He obtained his Ph.D. degree for studies concerning the synthesis of optically active arene chromium derivatives (1980). After a post-doc with C. A. Reed at the University of Southern California (Los Angeles, 1979), he returned to the Laboratoire of Chimie Organometallique. Since that time, he has been involved in teaching biochemistry at various levels at the University of Rennes 1. He is Director of research at the CNRS. His major research interests are the properties and reactivities of hemoproteins, their analogues, the synthesis of drugs for photodynamic therapy, and, recently, bioorganometallic chemistry. He is the author of over 130 publications and four book chapters.

Arnaud Bondon, born in 1960, studied Biochemistry and received his Ph.D. in Chemistry from the University of Rennes in 1987. After a postdoctoral stay, on cytochrome P450, at Vanderbilt University (with Prof. F. P. Guengerich), he returned to Dr. Simonneaux's group at the University of Rennes I as a Chargé de Recherche at the CNRS. He has been involved, in collaboration with Dr. J. Leroy, in the preparation and the evaluation of the catalytical activities of various β-fluorinated metalloporphyrins. However, his research was mainly focused on the hemeproteins with structural study of the active sites or electron-transfer analysis through NMR experiments. Particular attention was given to the study of paramagnetic systems. In 2004, he set up a new team (RMN–Interactions Lipides Protéines) in a biological unit of CNRS (UMR 6026) with the study of protein–lipid interactions as the main focus.

reviews resuming this approach.[17,26,29] Much of the effort has involved the use of spectroscopic techniques, although high-pressure,[30,31] cross-linking, potentiometric techniques and computational methods[32] have also been employed to investigate interprotein interaction.[33]

Another effective method for gaining insight into the electron-transfer mechanism is the use of NMR techniques. The pioneering work of Gupta and Redfield[34] on the determination of electron self-exchange of cytochrome *c* is a leading reference in this area. Since then, the electron-transfer self-exchanges of several other heme proteins have been analyzed.[35,36] Heme proteins (or iron porphyrins) are particularly suitable because heme proton resonances are often shifted outside the bulk of the diamagnetic protein resonances (0−10 ppm) by the strong ring current shift of the porphyrin ring and by isotropic shifts in the case of paramagnetic proteins. Thus, the electron-transfer rate determination can be studied by line broadening, magnetization transfer, or relaxation measurements. Examples of the methods will be described below, but first, we will present separately an overview of electron-transfer theory. Then, the main part of the review will focus on iron porphyrins, self-exchange, and interprotein and multi-heme protein electron transfers.

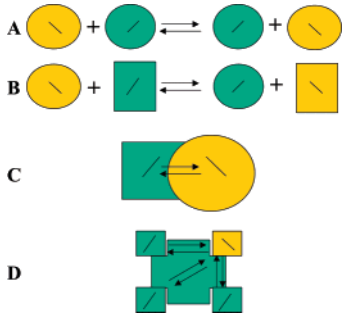

Figure 1. Schematic representation of self-exchange (A), cross-exchange (B), intracomplex (C), and intramolecular multiheme (D) electron transfers.

In addition, high-resolution NMR is a particularly useful method for the study of different redox states[37] and for the topological analysis of transient complexes,[38,39] since the chemical shifts observed in NMR spectra provide information on the electronic shielding of the observed nuclei. This complementary approach to the mapping of the protein−protein interaction will be presented in the interprotein electron-transfer section. Other widely used techniques to investigate homogeneous electron-transfer reactions, such as EPR (electron paramagnetic resonance)[40] or the recently reported MARY,[41] will not be detailed in this review.

The solution structures of heme proteins[42] and the use of NMR to characterize paramagnetic heme proteins[38,39,43−45] have been recently reviewed, and it is not our purpose to present a comprehensive literature survey in this area. To get complete coverage on NMR of heme proteins, it will be necessary for the reader to consult more specialized review articles.[37,43,46−53] An overview can also be obtained from the two recent reviews of Banci et al.[42] and La Mar et al.[44] This article will only emphasize use of NMR to study electron transfer in heme proteins. A schematic representation of self-exchange (A), cross-exchange (B), intracomplex (C), and intramolecular multiheme (D) electron-transfer reactions is shown in Figure 1.

2. Electron-Transfer Overview

To introduce the factors which control the rates of electron-transfer reactions, a brief overview, applicable to interprotein electron transfer, will be first presented. According to the Marcus formalism,[1] the

electron-transfer rate constant of a nonadiabatic reaction can be expressed as

$$k = \mathrm{A}\exp(-\Delta G^*/RT) \quad (1)$$

The term A depends on the nature of the electron-transfer reaction, e.g., bimolecular or intramolecular, and ΔG^* is the activation free energy. $A = \kappa Z$, where κ is the normalized probability of electron transfer to occur and Z is the collision frequency in an intermolecular electron-transfer reaction (or the vibrational frequency in an intramolecular electron transfer). The reorganization energy, λ, can be obtained through application of the following relationship:

$$\Delta G^* = (\lambda/4)(1 + \Delta G^\circ/\lambda)^2 \quad (2)$$

ΔG° is the standard free energy of the reaction. For the self-exchange reaction, $\Delta G^\circ = 0$. Therefore, the reorganization energy can be expressed as $\lambda = 4\Delta G^*$.

In a simple intramolecular system, k will decrease approximately with distance. This is reflected in eq 3, where the donor-to-acceptor distance is r, and r_0 is the close contact distance (3 Å).

$$k = 10^{13}\exp[-\beta(r - r_0)]\exp(\lambda/4)(1 + \Delta G^\circ/\lambda)^2/RT \quad (3)$$

The parameter β is used to quantify the nature of the intervening medium with respect of its efficiency to mediate electron transfer. Average values 0.7−1.4 Å^{-1} for β have been proposed for electron-transfer reactions.[54,55]

The simplified Marcus cross-relation[1] (eq 4) has also been applied to a number of electron-transfer reactions in heme proteins.

$$k_{12} = (k_{11}k_{22}K_{12}f_{12})^{1/2} \quad (4)$$

In practice, f_{12} is usually about equal to 1.[1] In this equation (eq 4), k_{12} and K_{12} are the rate and equilibrium constants for the cross-reaction, respectively, and k_{11} and k_{22} are the self-exchange constants. In this case, the theory proceeds from the assumption that, within the activated complex, the probability of electron transfer is unity and that the work terms for the self-exchange and cross-reactions are the same.[56]

Electron transfer between proteins is often associated with the formation of a complex of the reacting proteins prior to the electron transfer, with a possible configuration change in the protein, before the electron transfer. For a second-order reaction, the electron-transfer reaction can be divided into three steps: formation of the precursor complex D/A between the donor (D) and the acceptor (A) with an equilibrium constant K_{eq}, electron transfer (k_{ET}) to give the successor complex D^+/A^-, and dissociation to form the ions D^+ and A^-. A steady-state analysis of the system reduces the observed second-order rate to $k_{obs} = K_{eq}k_{ET}$ under favorable conditions.[57]

3. NMR Methods

3.1. The Classical Bloch–McConnell Equations

The NMR signals of chemically exchanging systems are described by the Bloch−McConnell equations.[58−60] There are many reviews on magnetization transfer,[60−63] and for brevity the theory will not be discussed in detail here. It has been shown[59,64] that, for a two site-exchange problem involving two sites A and B, eqs 5 and 6 applied:

$$\mathrm{A} \underset{k_\mathrm{B}}{\overset{k_\mathrm{A}}{\rightleftharpoons}} \mathrm{B}$$

$$\mathrm{dM}^\mathrm{A}_z(t)/\mathrm{d}t = [\mathrm{M}^\mathrm{A}_z(0) - \mathrm{M}^\mathrm{A}_z(t)]/T_{1\mathrm{A}} - k_\mathrm{A}M^\mathrm{A}_z(t) + k_\mathrm{B}\mathrm{M}^\mathrm{B}_z(t) \quad (5)$$

$$\mathrm{dM}^\mathrm{B}_z(t)/\mathrm{d}t = [\mathrm{M}^\mathrm{B}_z(0) - \mathrm{M}^\mathrm{B}_z(t)]/T_{1\mathrm{B}} - k_\mathrm{B}\mathrm{M}^\mathrm{B}_z(t) + k_\mathrm{A}\mathrm{M}^\mathrm{A}_z(t) \quad (6)$$

where $\mathrm{M}^\mathrm{A}_z(0)$ and $\mathrm{M}^\mathrm{B}_z(0)$ are the equilibrium magnetizations at sites A and B, respectively, $\mathrm{M}^\mathrm{A}_z(t)$ and $\mathrm{M}^\mathrm{B}_z(t)$ are the magnetizations at sites A and B at time t, k_A is the rate of transfer from site A to B, and T_{1i} is the spin−lattice relaxation time at site i = A, B in the absence of exchange. Equations 5 and 6 are known as the modified Bloch equations for a two-site exchange and describe the flow of magnetization. Although the validity of the approximate solutions of these equations generally applied, a complete analysis is often necessary, as has been underlined recently.[60,65] The different methods generally used for a system exchanging one electron will be first described below. Each of these methods has certain advantages and disadvantages, and this will also be considered. For an introduction to these methods, see, for example, the review and references therein previously reported by Gupta and Mildvan.[61]

3.2. Kinetic Measurements at the Equilibrium

All the methodologies for kinetic determination of the reaction rate were initially used for the analysis of the chemical interconversion of two species. The lifetimes of the species for a first-order reaction, which can be measured by different methods, are directly converted into reaction rates following the relations

$$\tau_\mathrm{A} = [\mathrm{A}]/k_\mathrm{A}[\mathrm{A}] = 1/k_\mathrm{A} \quad \text{and} \quad \tau_\mathrm{B} = [\mathrm{B}]/k_\mathrm{B}[\mathrm{B}] = 1/k_\mathrm{B} \quad (7)$$

It has to be emphasized that this lifetime is independent of the protein concentration and of the ratio of the two forms; this applies for intramolecular or inside stable binary complex electron exchange.

In the case of more complex reactions, the lifetime of a species reflects the sum of all the velocities responsible of the transformation of this species. Under self-exchange, which is a second-order reaction,

$$\mathrm{C} + \mathrm{D} \rightleftarrows \mathrm{D} + \mathrm{C} \quad (8)$$

the lifetime is $\tau_\mathrm{C} = 1/k[\mathrm{D}]$. To calculate the bimolecular rate constants, the total concentration of the protein and the percentage of D have to be known. For a general theory on dynamic NMR, the reader is advised to consult the book written by Drago.[66]

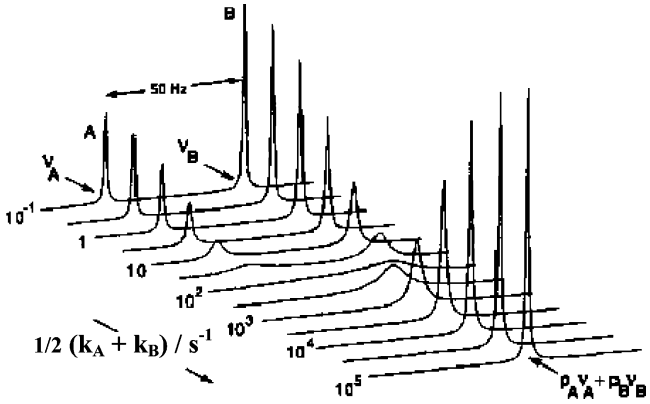

Figure 2. Schematic representation of a theoretical NMR spectrum of two exchanging species A and B as a function of the exchange rate.

In the case of exchange with one paramagnetic species, since the spin−lattice relaxation time of the electron is short ($T_{1e} \sim 10^{-12}$ s for Fe(III) complexes),[67] compared to the lifetime for the exchange process, the system can be considered as a two-site problem. Usually, the ratio of the oxidation states can be determined through integration of selected peaks in a 1D spectrum. The determination of the electron relaxation rates in paramagnetic metalloproteins has been very recently discussed.[68]

A large range of kinetics can be studied by ^{1}H NMR, ranging from 10^{-2} to 10^8 s^{-1}. However, the strategy to be used depends on the pattern of the signals in exchange, which is related to the difference in the Larmor frequencies of the two forms and the reaction rate. Slow exchange and fast exchange on the chemical shift time scale are two extreme conditions: the former refers to the presence of two different sets of resonances for the two proteins forms, the latter to the presence of resonances at a chemical shift that is the average of the chemical shifts of the two species, weighted for the molar fraction of the two forms. Line broadening of the resonances can be observed at all the intermediated situations. Whatever the rate, quasi slow or quasi fast, when the spectra are strongly affected, the rate of the reaction is related to the difference in hertz between the two states. This corresponds to the chemical shift time scale. In contrast, when the exchange is slow and line broadening cannot be detected, which corresponds to a lifetime of ~0.2 s, two other methods, spin−lattice relaxation time measurement or a saturation transfer experiment, can be successfully applied if the exchange rate is of the same order as the relaxation rate.

3.3. NMR Line Broadening

This method has been applied for both slow and fast exchange in the chemical shift time scale. In the case of fast exchange, the resonances of a given nucleus in the two oxidation states have coalesced into one resonance (Figure 2). Thus, the observed line width for two species in fast exchange is the weighted average of the natural widths of A and B plus an additional broadening due to the chemical exchange of the two species.[69] When neglecting T_2, the width of the resonance ($W_{red,ox}$) is given by

$$W_{red,ox} = \frac{f_{red} f_{ox} 4\pi(\delta\upsilon)^2}{c k_{ex}} \quad (9)$$

where $k_{ex} = k_A + k_B$, c is the total concentration, $\delta\upsilon$ is the difference in chemical shift between the two nonexchanging peaks expressed in hertz, and

$$f_{red} = \frac{\delta_{ox} - \delta_{obs}}{\delta_{ox} - \delta_{red}} \quad \text{and} \quad f_{red} = 1 - f_{ox}$$

Considering exchange between two low-spin ferric and ferrous heme derivatives (porphyrins or heme proteins), the averaged chemical shift difference of the heme methyl signals is roughly around 20−30 ppm. The limit for line broadening detection is defined as $2\pi\ \delta\upsilon \ll 1/\tau$; consequently, for such systems the lower limit for the electron exchange rate is of 10^4 order. The use of the heme methyl resonances for the lifetime determination is particularly relevant, because of the absence of spin−spin coupling, which is known to decrease the reliability of the measurements.[65,70]

In the case of slow exchange, this method applies when the rate induces a broadening of line resonances of the same spin under both states in exchange. In this situation, the exchange rate constant can be determined using the determination of the observed line broadening of each resonance.[61] However, this technique is not often used because it is very difficult to measure precisely the line broadening, and in the case of slow exchange in the chemical shift time scale, the lifetime determination is rather performed using relaxation time measurement or saturation transfer experiments.

3.4. Inversion Recovery Techniques

According to the classical theory of magnetization transfer, in the case of a moderately rapid reaction, the self-exchange rate constant can be determined by measuring the spin−lattice relaxation time.[61,71−74] This method is applicable only when a proton gives rise to two different nonoverlapping resonances in the two oxidation states and its lifetime in the oxidized state is long compared to the spin−lattice relaxation time in this state. The longitudinal relaxation time of one proton on the reduced state is measured in the absence and in the presence of the oxidized state. These measurements are performed using a conventional inversion−recovery pulse sequence (Figure 3).[72] Simplification of eqs 5 and 6 comprising the exchange terms is obtained when the lifetime of one of the two (oxidation) states (B) is long compared with the relaxation time.[61] Knowing these values in the reduced state enabled the lifetime to be estimated, using the equation

$$\frac{1}{T_{1app}^{red}} = \frac{1}{T_1^{red}} + \frac{1}{\tau_{red}} \quad (10)$$

where T_{1app}^{red} corresponds to the apparent T_1 of a proton on the reduced form, in the presence of the

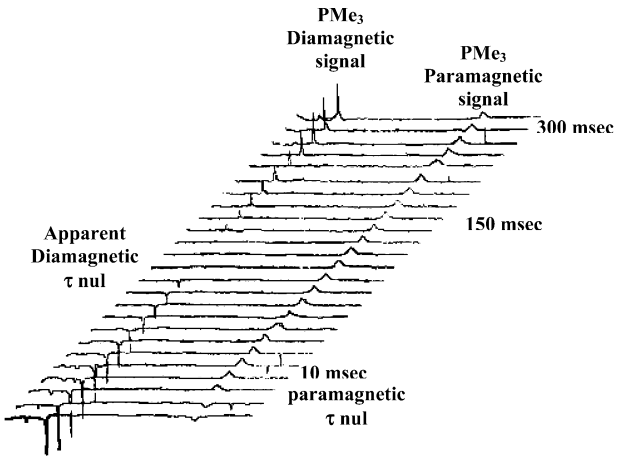

Figure 3. Example of relaxed ^{1}H NMR spectra of trimethylphosphine (PMe_3) horse heart myoglobin, as a function of τ in a 180°−τ−90° T_1 inversion−recovery experiment. The protons of the ligand were labeled around −12 ppm and −3 ppm for the oxidized and the reduced forms, respectively. Reprinted with permission from ref 72. Copyright 1992 Elsevier.

oxidized state. It must be emphasized that a selective or a nonselective pulse can be used. As expected, the selective pulse gives shorter T_1 values than the method in which all the peaks are inverted, due to the contribution of the cross-relaxation of the nuclei. However, it has been found that the two methods gave similar rate constants within the experimental error.[72] A method for determining unequal spin−lattice relaxation times in the presence of exchange has been reported.[75]

3.5. Saturation Transfer Experiments

Saturation transfer experiments can be used, when the exchange and relaxation rates are of the same order, to estimate the rate constant[61,63,64] and to assign hyperfine-shifted resonances of the paramagnetic oxidation state of a redox molecule.[76−79] This was first demonstrated in biological systems with electron exchange between ferri and ferro cytochrome *c* in 1970 by Gupta and Redfield.[34] If a strong radio frequency field is applied to site B, then $M^B_z(t) = 0$ and eq 1 becomes

$$dM^A_z(t)/dt = M^A_z(0) - M^A_z(t)/T_{1A} - M^A_z(t)/\tau_A \quad (11)$$

When the irradiation of B is long enough to reach steady state, $dM^A_z(t)/dt = 0$, and the ratio of the signal intensities of A in the presence (I_{on}) and the absence (I_{off}) of irradiation is related to the lifetime following this relation:

$$\frac{I_{on}}{I_{off}} = \frac{\tau_A}{\tau_A + T_1^A} \quad (12)$$

With this method, one should be able to measure the intensities of the signals in the reduced form with and without irradiation of the oxidized form. Furthermore, the longitudinal relaxation time T_1 of the signal in a fully reduced state has to be performed.

Since then, a number of optimizations of magnetization-transfer experiments for kinetic rate measurements have been reported.[80−82] Time-dependent increase of the magnetization transfer allows more accurate values of electron-transfer rate determination. Indeed, values of pure T_1 of the fully reduced signal can be fitted together with the transfer rate.[12,83]

3.6. Two-Dimensional Spectroscopy

The 2D EXSY spectrum has been introduced by Jeener and collaborators.[84] It corresponds to the NOESY sequence and permits the calculation of the exchange rate through the integration of the cross-peaks of the spectrum. 2D experiments are efficient for the detection of slow exchange between two sites; however, less precise estimates of rate constants are reported when compared with 1D methods. As long as experiments were conducted by irradiation of the most paramagnetic signal, 1D magnetization-transfer accumulation of exchange spin is under control of the reduced T_1. This is not true for the 2D experiments where the cross-peak intensities are under control of the T_1 of both species, as encountered for the selective 1D saturation transfer experiments. In the case of irradiation of a paramagnetic species, this is particularly relevant for the sensitivity of the experiment, because the relaxation rate of the paramagnetic signals is fast. So, 1D sequences have often to be preferred rather than EXSY spectra for the rate determination. Nevertheless, 2D EXSY spectroscopy is very useful in order to correlate spin resonances between the two exchanging species. This approach has been widely used in heme proteins through exchange of ligands or electron transfer.[79,85]

3.7. Mapping Protein−Protein Interaction by NMR

Several chemical shift mapping analyses of electron-transfer heme protein complexes have been reported.[86] There are recent reviews on this topic.[38,39] Nuclear Overhauser effect (NOE) can be used, in particular the isotope-edited NOE,[87,88] which discriminates between NOEs within a macromolecule and NOEs between two interacting macromolecules. This method is only applicable when the interaction between the molecules is tight ($K_d < 10$ μM) and requires different isotopic labeling patterns.[39] However, chemical shift perturbation is probably the most widely used NMR method to map protein interfaces. The ^{15}N−^{1}H HSQC spectrum[89] of one protein is monitored when the unlabeled interaction partner is titrated in the solution. The HSQC spectrum is a two-dimensional map that correlates the chemical shifts of the amide protons with the chemical shifts of the ^{15}N amide nitrogens. The interaction causes environmental changes on the protein interfaces and, hence, affects the chemical shifts of the nuclei in this area. The methods are well reviewed, recently.[90] There are other NMR methods such as cross-saturation, titration, mapping with dynamics, and mapping with pseudocontact shifts which are discussed in a review by Zuiderweg.[39] Most studies to determine the structure of complexes using the pseudocontact shift approach were carried out with heme proteins.[91−93] NMR studies of redox complexes involving at least one heme protein have been recently summarized.[44]

4. Self-Exchange Electron Transfers

The study of the electron self-exchange reaction is one effective way to demonstrate the electron mechanism because the thermodynamic driving force is zero in self-exchange kinetics. This simplifies the interpretation of the intrinsic electron-transfer process. Consequently, in its simplest form, the Marcus reorganization energy of a self-exchange reaction is directly given by the free energy of activation.[1] Thus, the theoretical interpretation of such a reaction is greatly simplified as compared to those of reactions with ΔG^* values different from zero, facilitating the study of other topics such as activation parameters or dynamic solvent effects on rate constants.[94] Differences in the self-exchange rate constants between similar proteins arise from differences in the electrostatic, geometric, and reorganizational characteristics of the proteins. Thus, bimolecular electron transfers in biological systems, which occur between species that usually have a net charge as well as a substantially asymmetric charge distribution, are easier to study in self-exchange reactions, since we are concerned with the same protein in two different redox states. In addition, in exchange reactions, the molecular properties of only one redox couple rather than of two different redox couples need to known.[1] Given an appropriate model, one can use the dependence of the rate constant on ionic strength to calculate the work term at a given ionic strength and the rate constant extrapolated to infinite ionic strength. The theoretical Van Leeuwen approach[16] was found to be the best at the ionic strengths used in NMR experiments.[95,96] It is also possible to predict the rate constants of mixed redox reactions using the Marcus cross-relation (eq 4).

4.1. Iron Porphyrins

^{1}H NMR techniques have proved to be a useful approach to study electron-transfer reactions between iron porphyrins. Actually, whereas numerous studies have been performed on heme proteins in order to determine the factors which control the rate of electron transfer, only a few experiments have been carried out directly measuring self-exchange electron transfer between hemes.[36] The ^{1}H NMR investigations of Dixon and co-workers were particularly important in this area, using ^{1}H NMR line broadening analysis,[97−100] one of the limits being the complexity of the natural porphyrin proton spectra.[98] However, ^{1}H NMR line-broadening measurements have been shown to be much more reliable than measurements obtained by cross-reaction between heme and inorganic reagents.[101−104] All the measured rate constants by ^{1}H NMR range from 10^7 to 10^8 M^{-1} s^{-1} (Table 1). Thus, the changes in the substituent pattern on the porphyrin macrocycle have relatively little effect on the k values for different tetraphenylporphyrins. However, the complex with axial imidazole (unsubstituted N−H) has a self-exchange rate constant smaller by a factor of 2−3 than those with N-alkyl substituents.[99] This indicates that the N−H bond may play a role in controlling electron transfer. The difference was ascribed to stronger hydrogen bonding in the N−H in the Fe(III) as compared with that in the Fe(II) species. Consequently, an increase in the outer sphere reorganization energy is observed, and this leads to a decrease of the rate.[1,105] In contrast, the inner sphere reorganization is small (<1 kcal/mol) because the structure of the porphyrin complex changes little on going from Fe(II) to Fe(III).[106,107] Outer-sphere reorganization energies in different iron porphyrins were calculated by Dixon et al.[98] using the equation[5]

$$\lambda_{out} = (e^2/2r)(1/D_0 - 1/D_S) \quad (13)$$

where r is the radius of the reactant ion and D_0 and D_S are the optical and static dielectric constants, respectively. The λ_{out} values estimated for the dicyano complex were 2.9 and 3.6 kcal/mol in DMSO and methanol, respectively.[98] A microscopic treatment of the energetics of electron-transfer reactions in aqueous environments and proteins has been realized.[108] Accordingly, the results indicated that, compared to a polar solvent, the protein undergoes a small dielectric relaxation upon oxidation of the heme.

Incorporation of fluorine atoms in biological molecules permits a dramatic simplification of the spectra, and this advantage has been explored with many different biological systems,[109] in particular with artificial heme proteins.[110−112] Thus, ^{19}F NMR can also be used to determine the self-exchange transfer rate, between ferric and ferrous porphyrins, using difluorovinyl analogues of natural hemin such as iron bis(difluorovinyl) deuterioporphyrins.[111] Actually, this derivative was first synthesized and tested as a photosensitizer for photodynamic therapy.[113] The large variations of chemical shifts depending of the oxidation state permitted the measurements of the electron self-exchange rate constants of bis(1-methylimidazole)iron complexes in various solvents by analysis of the line broadening of the ^{19}F NMR signals (Figure 4).[111] The experimental rate constants were strongly affected by the nature of the solvents, varying from 3.9×10^7 to 24×10^8 M^{-1} s^{-1} for DMSO and acetone, respectively. These measured rate constants are in agreement with previously reported values for other iron porphyrins.[97]

Table 1. Examples of Electron-Transfer Self-Exchange Rate Constants of Iron (II)/(III) Porphyrins Determined by NMR

oxidized form of the system	solvent	T (K)	k_{ex} (M^{-1} s^{-1})	ref
(TPP)Fe(1-MeIm)$_2^{7+}$	CD_2Cl_2	252	8.1×10^7	97
(3-MeTPP)Fe(1-MeIm)$_2^{7+}$	CD_2Cl_2	252	5.3×10^7	99
(4-MeTPP)Fe(1-MeIm)$_2^{7+}$	CD_2Cl_2	252	9.7×10^7	99
(4-OMeTPP)Fe(1-MeIm)$_2^{7+}$	CD_2Cl_2	252	6.8×10^7	99
(2,4,6-Me$_3$TPP)Fe(1-MeIm)$_2^{7+}$	CD_2Cl_2	252	1.6×10^8	99
(3-TPP)Fe(5-MeIm)$_2^{7+}$	CD_2Cl_2	252	1.6×10^7	94
(2,4-difluorovinyl-	$CDCl_3$	295	18.3×10^7	111
DPDME)Fe(1-MeIm)$_2^{7+}$	DMSO	295	3.9×10^7	111
	$(CD_3)_2CO$	295	24.1×10^8	111
(TPP)Fe(CN$^-$)$_2^{7-}$	DMSO	310	5.8×10^7	98
(3-MeTPP)Fe(CN$^-$)$_2^{7-}$	DMSO	310	3.4×10^7	98
(4-MeTPP)Fe(CN$^-$)$_2^{7-}$	DMSO	310	4.4×10^7	98
(4-OMeTPP)Fe(CN$^-$)$_2^{7-}$	DMSO	310	2.9×10^7	98
(4-*i*-PrTPP)Fe(CN$^-$)$_2^{7-}$	DMSO	303	3.1×10^7	98
(DPDME)Fe(CN$^-$)$_2^{7-}$	DMSO	303	1.0×10^7	98
(PPDME)Fe(CN$^-$)$_2^{7-}$	DMSO	303	1.5×10^7	98
(TPP)Fe(CN$^-$)$_2^{7-}$	CD_3OD	310	1.6×10^7	98

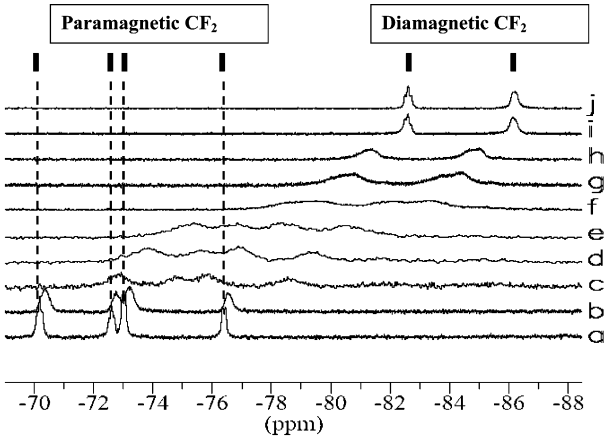

Figure 4. ^{19}F NMR spectra of equal amounts of bis(1-MeIm)(2- and 4-)difluorovinyldeutero porphyrin iron complexes in DMSO at 295 K: (a) fully oxidized; (b−i) partially reduced; (j) fully reduced (adapted from ref 111).

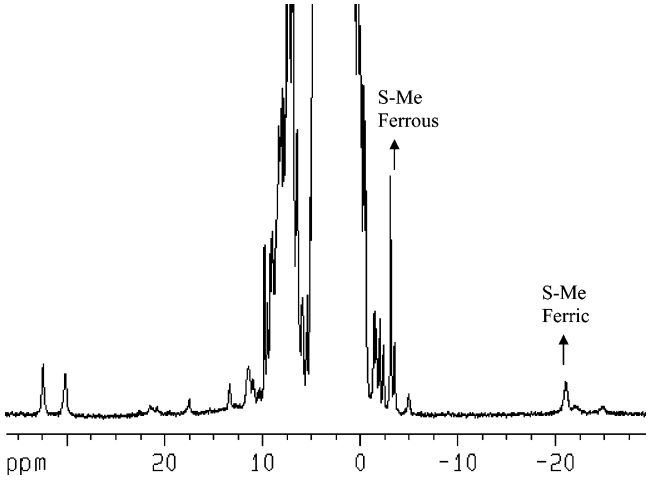

Figure 5. ^{1}H NMR spectrum of a 70/30 ferric/ferrous mixture of horse heart cytochrome *c* in D_2O at 313 K (pD 7).

The self-exchange rate constants of other metalloporphyrins have also been determined. Thus, the direct determination of the small rate constants of cobaltoporphyrins has been made by measuring the rate of electron transfer from methyl-labeled tetraphenylporphyrin cobalt(II) to the cobalt(III) chloride complex in the presence of pyridine, because the exchange is slow enough[114] to monitor the reaction by ^{1}H NMR as a function of time.[103] Electron-transfer reactions of metalloporphyrins can also involve the porphyrin ligand, which can be redox active. In this case, the values are very high, due to low reorganization energy.[115]

4.2. Heme Proteins

Numerous self-exchange reactions have been studied in order to understand electron transfer in heme proteins.[36,96,116,117] It is an approach to probe the factors that control electron transfer from measurement of self-exchange between the oxidized and reduced forms of the same protein. They can often be measured directly by NMR spectroscopic techniques, by either NMR saturation transfer, spin−lattice relaxation, or line shape measurements, depending of the value of the exchange rate. Actually, two extreme situations were observed:

(1) The spectrum of mixed oxidation states is not the simple superposition of individual ferri and ferro spectra. Such behavior has been taken to indicate rapid chemical exchange between the two forms due to rapid electron transfer, and the rate constant is determined by line shape measurements. For example, in the case of *Pseudomonas* cytochrome c_{551}[118] and cytochrome c_{554} from *Alcaligenes faecalis*,[119] mixed-state spectra have been used to measure an electron-transfer exchange rate of 1.2×10^7 M^{-1} s^{-1} and 3×10^8 M^{-1} s^{-1}, respectively.

(2) Mixtures of ferric and ferrous cytochromes give NMR spectra that are the simple superposition of a pure ferric and a pure ferrous component. An example of the ^{1}H NMR spectrum of a 70/30 ferric ferrous mixture of horse heart cytochrome *c* in D_2O at 313 K (pH 7) is shown in Figure 5. In this case, the rate of electron transfer can be determined by saturation transfer or relaxation time measurements, as initially reported by Gupta.[61,71]

4.2.1. Cytochromes

The self-exchange constants of various cytochromes are summarized in Table 2. These rate constants span at least 6 orders of magnitude, from 10^2 to 10^8 M^{-1} s^{-1}. Numerous self-exchange reactions have been studied by ^{1}H NMR with horse heart cytochrome *c*, with native,[71,120] site-specific chemical modification,[121] and axial ligand exchange.[73] However, the rate with cobalt cytochrome *c* is too slow to be determined by ^{1}H NMR after metal substitution (iron by cobalt).[122] A study of the electron-transfer properties of the heme undecapeptide from horse cytochrome *c*, which has been used as a model of cytochrome *c*,[123] has also been reported.[124] The self-exchange rate constant is 1.3×10^7 M^{-1} s^{-1} at 330 K.[124] The value for horse heart cytochrome *c* is much slower (Table 2). Two factors were proposed to explain this difference: the heme is less exposed to the solvent, and the protein surface is more highly charged (positive) around the exposed part in the intact protein by comparison to the situation observed in the heme undecapeptide.[124]

Cytochrome *c* is a ubiquitous soluble heme protein localized in the intermembrane compartment of the mitochondria. However, interaction of this protein with the inner mitochondrial membrane has been highlighted a long time ago.[125−127] Under physiological conditions, a small fraction of cytochrome *c* is bound to the inner mitochondrial membrane and has been associated with a modulation of the electron-transfer rate.[128,129] As a model study, an estimate of the self-exchange electron-transfer rate of the micellar cyano cytochrome *c* has recently been obtained by NMR.[130] Surprisingly, a rate constant of 2.5×10^4 M^{-1} s^{-1} was found, close to the value of the native cytochrome *c* under similar condition. As an example of such an electron transfer, a 2D EXSY spectrum of a partially reduced cytochrome *c* Fe(III)CN solution in the presence of micelles is shown in Figure 6. The strong cross-peak correlations between the two redox forms of the heme methyl proton through electron self-exchange are labeled.[130] Although sodium dodecyl

Table 2. Examples of Electron-Transfer Self-Exchange Rate Constants of Heme Proteins Determined by NMR

system	salt (M)	T (K)	k_{ex} (M^{-1} s^{-1})	ref
heme undecapeptide	0.5 NaCN	330	1.3×10^7	124
cytochrome c_{550}				
Thiobacillus versutus WT	0	298	2×10^2	135
Thiobacillus versutus WT	0.55 NaCl		1×10^5	135
K14Q	0	298	7×10^3	135
K14E	0	298	1.2×10^4	135
Paracoccus denitrifans	0.1 NaCl	298	1.6×10^4	136
cytochrome c_{551}				
Pseudomonas aeruginosa WT	0.05 KP	315	1.2×10^7	118
Pseudomonas aeruginosa WT	∞[a]	298	2×10^7	96
Pseudomonas aeruginosa V23D	0.05 KP	313	2.8×10^4	12
Pseudomonas stutzeri	0.05 KP	313	4×10^7	303
cytochrome c_{554} *Alcaligenes faecalis*	0.1 NaCl	298	3×10^8	119
cytochrome *c* horse heart				
WT	0.1 KCl, KP		5×10^4	120
WT	∞	298	5.1×10^5	96
CDNP-K13	0.24 cacodylate	298	6×10^3	121
CDNP-K72	0.24 cacodylate	298	6×10^4	121
PMe_3 liganded	0.1 KP	298	7.5×10^3	73
PMe_3 liganded	∞	298	3.9×10^5	73
cytochrome *c Candida krusei*	0.1 KP	313	1×10^2	146
iso-1-cytochrome *c Saccharomyces cerevisiae*	0.1 KP	303	1×10^4	304
myoglobin PMe_3 horse heart	0.1 KP	296	3.1×10^3	72
myoglobin PMe_3 horse heart	0.7 KCl	296	3.9×10^3	72
myoglobin PMe_3 horse heart	∞	296	7.5×10^3	74
hemoglobin PMe_3 human α/α	0.1 KP	296	3.2×10^3	157
hemoglobin PMe_3 human *β*/*β*	0.1 KP	296	2.09×10^3	157
cytochrome b_5	0.1 KP	298	2.6×10^3	35
cytochrome b_5	1.5 KP	298	4.5×10^4	35
cytochrome b_5	∞	298	3.7×10^5	35
flavocytochrome b_2		298	2.3×10^6	143

[a] The symbol ∞ means extrapolated rate at infinite ionic strength: WT, wild type; KP, potassium phosphate buffer.

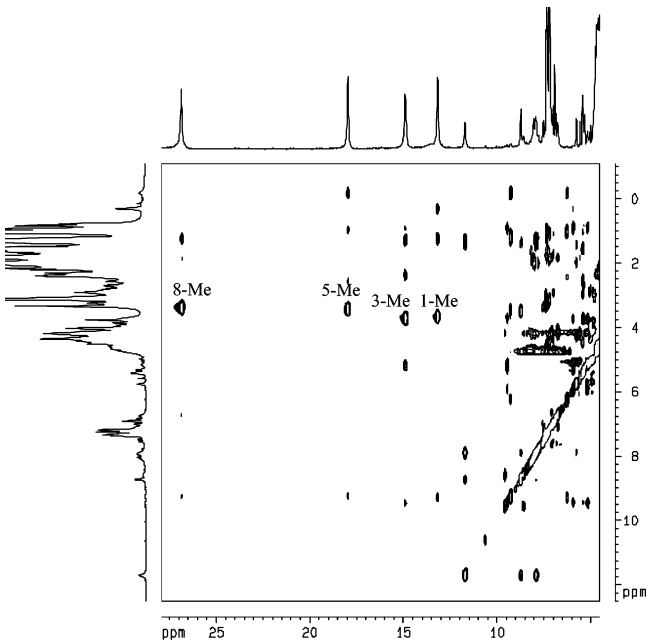

Figure 6. 2D EXSY spectrum of a partially reduced cytochrome *c* Fe(III)CN solution in the presence of micelles. The strong cross-peak correlations between the two redox forms of the heme methyl protons through electron self-exchange are labeled (adapted from ref 130).

sulfate (SDS) micelles have been extensively studied using various methods,[131−133] a more complete NMR study is still needed to elucidate the dynamic behavior and the mechanism of the electron transfer in this system.[130] A structure of a molten globule protein with altered axial ligation was recently suggested for cytochrome *c* and SDS from a careful 2D NMR analysis.[134]

Cytochrome c_{550} is a close relative of cytochrome *c* from mitochondria,[135] and several NMR studies of cytochrome c_{550} have been reported for *Thiobacillus versutus*[135] and *Paracoccus denitrificans*.[136] The electron-transfer self-exchange rates of wild type and mutants of the former have been determined by saturation transfer, T_1, and T_2 experiments.[135] For the wild type, at the zero ionic strength, the rate is 2×10^2 M^{-1} s^{-1} while it is 1×10^5 M^{-1} s^{-1} in the presence of 0.55 M NaCl. This result was expected, since the ring of positive charges around the heme crevice decreases the rate at low ionic strength. Few differences in the rates were observed between the two species due to small structural differences between the two cytochromes c_{550}.

Cytochrome c_{551} from *Pseudomonas aeroginosa* is a monomeric redox protein of 82 amino acid residues with His-Met ligation, involved in dissimilative denitrification. There are several studies of cytochrome c_{551} by NMR,[118,137,138] and the three-dimensional structure has also been determined in solution.[139] This protein, which carries a lower net charge than that of cytochrome *c* at physiological pH, has an electron-transfer self-exchange rate considerably higher[118,138] than that of cytochrome *c* (Table 2).[96] To map the surface involved in electron transfer, specific mutations in residues belonging to the hydrophobic patch were introduced by Brunori and co-workers.[13] It was found that introduction of negative charge in this area severely hampers both heteronuclear and homonuclear electron transfer, decreasing the self-

exchange value from 10^7 M^{-1} s^{-1} (native) to 2.8×10^4 M^{-1} s^{-1} (Val23Asp mutant).[13] Reasonable agreement of a theoretical study of the dynamics of the self-exchange electron transfer with kinetic data from NMR was obtained by Herbert and Northrup.[140]

There are some NMR studies on cytochrome c_{554}, which is a mono-heme c-type protein.[119,141] The self-exchange electron-transfer rate constant was estimated to be 3×10^8 M^{-1} s^{-1} for cytochrome c_{554} from the bacterium *Alcaligenes faecalis*.[119] This is one of the largest self-exchange rates observed for a cytochrome *c* (Table 2), due probably to few electrostatic interactions, a relatively low molecular weight (86 amino acids), and small reorganizational energy.

Flavin−protein interaction in flavocytochrome b_2 has been studied by NMR after reconstitution of the enzyme with ^{13}C- and ^{15}N-labeled flavin.[142] Using NMR spectroscopy, an electron self-exchange rate constant was determined for flavocytochrome b_2 of 2.3×10^6 M^{-1} s^{-1},[143] which is more than 2 orders of magnitude larger than the value obtained for microsomal cytochrome b_5, a homologue of a cytochrome b_2 domain.

Electron-transfer reactions with cytochrome b_5, which is a small (82 amino acids after tryptic digestion),[144] structurally well characterized protein,[145] have also provided a test for protein−protein electron transfer.[12] The self-exchange rates of cytochrome b_5 mutants are relatively slow on the NMR time scale, although the heme is far more exposed to solvent than that of cytochrome *c*.[35]

These large differences have been rationalized on the basis of Marcus reorganization energy,[116,138] different size, dipole moment, and heme exposure,[96,116] electrostatic charges, and polypeptide chain.[146] Brownian dynamics has also been employed to simulate the kinetics of the electron-transfer self-exchange reactions of trypsin-solubilized bovine liver cytochrome b_5 and horse heart cytochrome *c*, and it was found that the smaller reorganization energy of the cytochrome *c* plays the dominant role.[117] The small changes in the bond lengths, determined from X-ray absorption fine structure data,[147] are also consistent with the small reorganization energy[108,148] for the relatively fast electron-transfer reaction of cytochrome *c*. This result provides further support for the proposal that proteins assist in electron-transfer reactions by comparing the reorganization energies of the prosthetic groups in proteins to the reorganization energies of the same prosthetic groups at the same distance in water.[148] Early suggestions that electron transfer occurs mainly through the exposed heme edge in bimolecular electron-transfer reactions[10] were confirmed by various experiments involving derivatization of specific residues on the protein surface.[116] To delineate more precisely the factors that control electron transfer, the self-exchange reactions of three heme proteins—cytochrome *c*, cytochrome c_{551}, and cytochrome b_5—were analyzed, in terms of Marcus theory and molecular modeling to calculate heme−heme distance.[116] These latter values, together with the experimental rate constants as a function of temperature and ionic strength, give the reorganizational

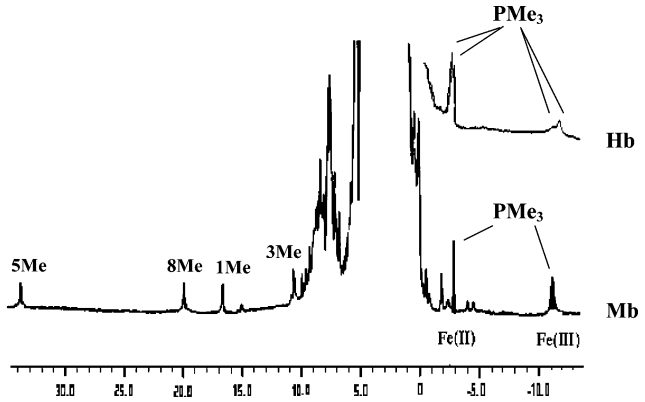

Figure 7. ^1H NMR spectrum of a 70/30 ferric/ferrous mixture of horse heart myoglobin metMbPMe$_3$/MbPMe$_3$ in D_2O (pD 6.9). Resonances of the proton of the PMe$_3$ are labeled. This inset shows the corresponding human hemoglobin adduct for which two sets of signals are observed in the two redox states. Reprinted with permission from ref 72. Copyright 1992 Elsevier.

values, λ, which are 0.7, 0.5, and 1.2 eV for cytochromes *c*, c_{551}, and b_5, respectively. The van Leeuwen approach[16] was used to calculate the interaction energy for two proteins in a heme edge-to-heme edge geometry, presumed to be the favored geometry for electron transfer. The work terms were 2.7 and 3.1 kcal/mol for cytochrome *c* and cytochrome b_5, respectively.[35]

4.2.2. Myoglobin and Hemoglobin

Even though myoglobin is not directly involved in electron-transfer reactions, an understanding of the mechanism of a self-exchange reaction involving this heme protein is important in the understanding of the overall picture of electron-transfer processes in heme proteins.[72] Fewer kinetic studies of redox reactions of the oxygen carriers, hemoglobin and myoglobin, have been undertaken,[149−152] in part because suitable reduced and oxidized states have not been available.[153] These studies have focused on the oxidation−reduction pathways of myoglobin (or hemoglobin) with a variety of organic or 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,[150,154] and no direct experimental determination was possible before complexation of myoglobin by trimethylphosphine.[72,155]

Several conditions make the trimethylphosphine/myoglobin system an excellent candidate for the study of self-exchange electron-transfer reactions. First, trimethylphosphine may serve as a ligand of both the ferric[156] and the ferrous heme of myoglobin (Figure 7). Second, this phosphine-ligated myoglobin is particularly amenable to study via NMR techniques because of the presence of a well-resolved upfield-shifted methyl resonance of PMe$_3$ which is coordinated to the metal atom in both oxidation states. Inversion recovery techniques were used to measure the spin−lattice relaxation time and to

calculate the self-exchange rate constant. For horse heart myoglobin, the rate is 3.1×10^3 M^{-1} s^{-1} at 296 K in 0.1 M phosphate at pH 6.9. The rate was weakly dependent on ionic strength with up to 0.7 M added KCl in solution, in contrast with a previous report with cytochrome *c*. It should be noted that the self-exchange of electron transfer for horse heart myoglobin is slow by comparison to the rate for native cytochrome *c* (vide supra). To assess the role of this exogeneous ligand (PMe_3), the rates of self-exchange electron transfer in the trimethylphosphine complex of cytochrome *c* have been measured by an NMR method over a large range of ionic strengths using inversion−recovery.[73,74] The rate is 1.56×10^4 M^{-1} s^{-1} at 296 K at pH 6.9 (μ = 0.34 M). The ionic strength dependence of the rate constant was treated by the van Leeuwen theory,[16] and extrapolation of the rate constant to infinite ionic strength gives a rate constant of 3.9×10^5 M^{-1} s^{-1}. The rate values of these systems range over 2 orders of magnitude with myoglobin PMe_3 ≪ cytochrome b_5 < cytochrome *c* PMe_3 < cytochrome *c*.

Like myoglobin, hemoglobin is also able to ligate PMe_3 in both the ferrous and ferric state (Figure 7).[156−158] Thus, ^{1}H NMR studies of PMe_3 bound to ferric hemoglobin are very characteristic, since the spectrum exhibits two resonances at very high field: −12.4 ppm for the β subunit and −13 ppm for the α subunit. As an application, an experimental investigation of the intermolecular mechanism of the electron transfer in hemoglobin is possible. Using saturation transfer experiments with native and [Fe, Mn] hybrid Hb, it was shown that both intra- and interchain electron transfer occur. Calculations based on ^{1}H NMR spectra and using the inversion−recovery method led to the following intermolecular rate constants: $k_{11} = 3 \times 10^3$ M^{-1} s^{-1} (bimolecular α−α chain electron transfer) and $k_{22} = 2 \times 10^3$ M^{-1} s^{-1} (bimolecular β−β chain electron transfer) for self-exchange electron transfer and $k_{12} = 1 \times 10^3$ M^{-1} s^{-1} {[α(II)PMe_3] + [β(III)PMe_3]} and $k_{21} = 0.4 \times 10^3$ M^{-1} s^{-1} {[α(III)PMe_3] + [β(II)PMe_3]} for heterosubunit transfers. A plot of lifetime versus the inverse of ferric hemoglobin concentration shows a good linear relationship according to a bimolecular reaction without any significant intramolecular contribution. An application of the Marcus relationship to the cross-reactions yields $k_{12} = 3 \times 10^3$ M^{-1} s^{-1} and $k_{21} = 1.7 \times 10^3$ M^{-1} s^{-1} values, which are in good agreement with the NMR values. 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 10. This indicates that these electron transfers represent reactions by a single pathway with no evidence of configurationally limited behavior. It is also of interest to point out that the difference in chain concentration in both iron redox states corresponds to a difference in oxidation−reduction potentials which is 21 mV. Accordingly, the oxidation−reduction equilibrium of the isolated α and β chains[159] and of haptoglobin-bound hemoglobin[160] showed a difference in the redox potentials of 61 mV and 56 mV, respectively. In this system, self-exchange electron transfer and cross-exchange electron transfer (see section 6) are observed without any intraprotein electron transfer (see section 5).

5. Multiheme Intraprotein Electron Transfer

Interprotein electron transfers occur in general through the formation of binary or ternary complexes. These entities display very variable stability depending on the extent of the interactions between the proteins (see section 6).[4] The proteins involved in electron transfer are often charged, and the resulting electrostatic interactions contribute to the specificity of the association between redox partners. As a consequence, the systems studied by NMR, when they are electron-transfer reactions induced by collisions, correspond mainly to self-exchange reactions. A different situation is observed with multiheme proteins, which often display two mechanisms of electron transfer, one intramolecular and one intermolecular; the latter can be viewed as a self-exchange reaction for the intermolecular part of the reaction. These proteins can be considered as a good example of the use of an NMR methodology (i) to solve the redox potential of the individual hemes[161] and consequently to evaluate the heme−heme redox interactions and (ii) to estimate both the intramolecular and the intermolecular electron-transfer rate constants. Multiheme cytochromes have been extensively studied both biochemically and structurally, and a possible classification has been proposed.[162] We will focus in this section on the dynamics of electron transfer, and some characterization of the interaction between two redox partners will be described in section 6. For more general information on NMR studies of paramagnetic heme proteins, see recent papers reported by Bertini et al.[37,42] and La Mar et al.[44] Many reviews have also been previously reported on electron transfer in multicenter redox proteins.[29,44,161,163−165]

5.1. Cytochromes c_3

Cytochromes c_3 have been widely characterized by ^{1}H NMR since the first report in 1974 by McDonald et al.[166] X-ray crystal structures of several cytochromes c_3 from organisms such as *Desulfovibrio gigas,*[167] *Desulfovibrio vulgaris* Hildenborough[168] and Miyazaki,[169] *Desulfovibrio desulfuricans*,[170] and *Desulfomicrobium baculatum*[171] have been solved, showing that overall protein folding is conserved. Many of these heme proteins from different strains have also been characterized by NMR.[172−175] They are tetraheme iron proteins of molecular weight around 13 000 with low redox potential and are present in all the anaerobic sulfate-reducing bacteria belonging to the *Desulfovibrio* genus.[163] The iron atoms are coordinated by bis-ligated histidines and a *c*-type heme. With some exceptions, such as *Desulfovibrio desulfuricans* Norway,[176] cytochromes c_3 are characterized by a fast, in the NMR chemical shift time scale, intramolecular electron-transfer rate. This induces a single chemical shift for the heme methyl resonances of each heme whatever the localization of the reduced iron among the four hemes of the protein. So, for each oxidation state, a set of reso-

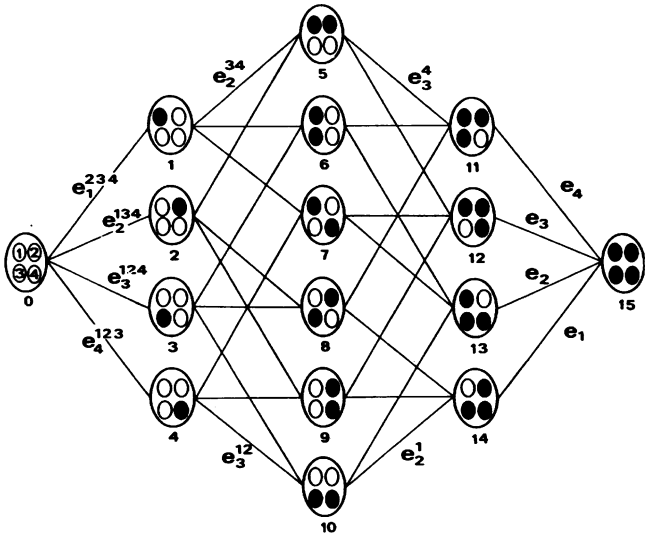

Figure 8. Diagram showing the sixteen populations that correspond to the five redox states connected by one electron step in cytochrome c_3 (adapted from ref 178).

nances is observed for each heme. A careful analysis of the observed chemical shifts at various levels of reduction permits assignment of the heme methyl resonances of the individual heme and calculation of their redox potentials depending on the redox state of the whole protein. In two pioneering works, Moura et al.[177] and Santos et al.[178] showed that NMR spectroscopy can be used to estimate the relative difference in the microscopic redox potentials and apply it to cytochrome c_3 from *Desulfovibrio gigas*. A diagram showing the 16 populations that correspond to the five redox states connected by one electron step in cytochrome c_3 is shown in Figure 8.[178] Since then, other extensive NMR methodological analyses for cytochrome c_3 of *Desulfovibrio vulgaris* have been reported.[179−181] Examples of saturation transfer NMR experiments to follow intermolecular reactions are shown in Figure 9.[182] EPR results have also been used to determine the four macroscopic redox potentials after redox titration.[183] However, in the particular case of *Desulfovibrio desulfuricans,* the assignment deduced from the ordered microscopic heme redox potentials determined by EPR[184] was in disagreement with that reported by NMR.[173] From the kinetic point of view and based on the chemical shift difference between the reduced and the oxidized states, only a lower limit of the electron-transfer rate, about 10^5 s^{-1}, is accessible by ^{1}H NMR. As pointed out in section 3, when the rate of the reaction is slow in the NMR chemical shift time scale, it is not possible to determine directly the rate constants by normal spectra analysis. The intermolecular rate constants can however be obtained using one-dimensional saturation transfer experiments or two-dimensional EXSY spectra. These experiments allowed the assignment of the heme methyl resonances of each heme for the various redox states of the protein. Depending on the cytochrome, the redox difference between the individual heme, the electron-transfer rate, and the redox state of the protein, up to four states can be detected for one irradiation, as observed with cytochrome c_3 of *Desulfovibrio vulgaris* Miyazaki F.[179−181] This has been successfully applied

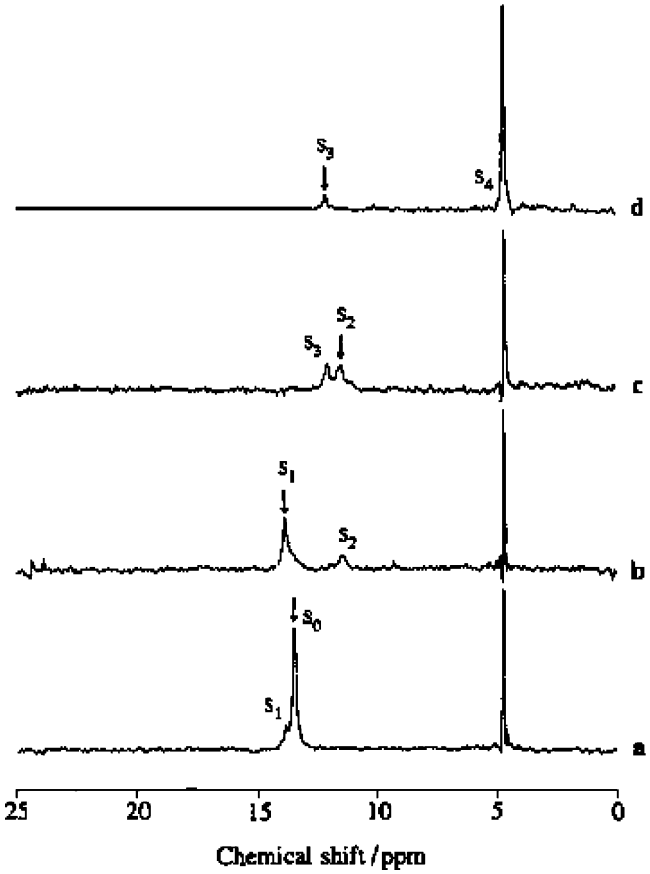

Figure 9. Saturation transfer NMR difference spectra of a methyl-2 (heme 3) in cytochrome c_3 (303 K, pD 7). The irradiated positions are indicated with arrows. S0, S1, S2, S3, and S4 stand for fully oxidized, one-electron reduced, two-electron reduced, three-electron reduced, and fully reduced, respectively (adapted from ref 182).

for different cytochromes c_3, and the calculated rates are in the range of 10^4 M^{-1} s^{-1}. The directionality of the stepwise electron transfer in cytochrome c_3, which may affect the functionality, was probed by site-specific mutagenesis.[185]

The previous NMR methodology has recently been extended to large cytochromes c_3, such as flavocytochrome c_3 from *Shewanella frigidimarina*.[186] As expected, when compared with small multiheme cytochromes c_3, the much larger protein shows broader NMR signals due to the slower tumbling rate of the molecule. Nevertheless, the 2D NMR spectra obtained in partially oxidized samples show that, on the NMR time scale, this cytochrome exhibits fast intramolecular and slow intermolecular electron transfer between the four hemes.

5.2. Cytochrome c_4

Different soluble diheme cytochromes c_4 have been characterized by NMR,[187,188] EPR,[189,190] electrochemistry,[191] and X-ray structure.[192] The NMR redox pattern observed for this cytochrome indicates that the electronic exchange process, which is dominated by intermolecular exchange, is intermediate to slow on the NMR time scale.[187] Interaction of cytochrome cd_1, which is also a diheme protein, with ferrocytochrome c_{551} has been followed by ^{1}H NMR.[193]

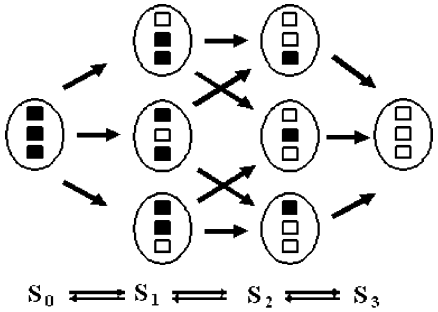

Figure 10. Model for the electronic distribution in a triheme protein. The inner rectangles represent the three heme groups that can be reduced (black rectangles) or oxidized (open rectangles). E_1, E_2, and E_3 are the macroscopic potentials connecting the four stages of oxidation (S_{0-3}) (adapted from ref 200).

5.3. Cytochrome c_7

Cytochrome c_7 is a triheme cytochrome isolated from *Desulfuromonas acetoxidans*. It is a member of the cytochrome c_3 family[194] but has a deletion in the protein segment that binds heme II.[195] The solution structure of the three-theme core has been determined by ^{1}H NMR,[196−198] and the X-ray crystallographic structure has been recently obtained.[199] At the concentrations typically used for NMR spectroscopy, cytochrome c_7 undergoes intermolecular electron exchange at a rate that is faster than that observed for tetraheme cytochrome c_3 from *Desulfovibrio*, and fast in the NMR time scale.[195] To slow the intermolecular electron transfer down to a rate in the intermediate to slow region on the NMR time scale, it was necessary to increase the ionic strength (0.5 M KCl) and decrease the temperature (289 K). Under these conditions, each heme substituent displays four discrete NMR signals, corresponding to each of the four redox stages. The thermodynamic properties were determined through the fit of redox titration data, followed by NMR and visible spectroscopy, to a model of four functional centers. After determination of the kinetics of sodium dithionite reduction of the three hemes, analysis indicates that the electrons enter the cytochrome mainly by heme I.[195] Specific assignments of NMR resonances to individual heme protons of the *Geobacter sulfurreductens* triheme cytochrome c_7 allowed the comparison of the spatial arrangement of the heme core in solution[200] and in the crystal. A model for the electronic distribution was proposed according to a redox titration followed by NMR and visible spectroscopy (Figure 10).[200] This theoretical model has been successfully applied to the determination of macroscopic reduction potentials of several multiheme cytochromes.[201]

6. Interprotein Electron Transfer

The development of 2D and 3D NMR methods and their application to labeled proteins has opened the field of interprotein interactions.[202] Transient protein complexes[203] are formed when high turnover is required, such as in redox processes occurring in cellular metabolism.[17,204] The kinetics of electron transfer between two different proteins can be determined using a variety of methods, depending on the time scale required.[4] A number of redox protein complexes of either physiological on nonphysiological partners have been investigated by NMR methodology.[205−207] Information regarding the kinetics,[208,209] the solution structure of transient complexes,[92,93] and the molecular recognition sites can be obtained. We will focus herein on protein complexes, in which the association involves at least one heme protein. Since many different systems exist, we will focus on electron-transfer complexes in which the interaction was significantly studied by NMR. Other cases have partly been described in a recent review.[44]

6.1. Cytochrome *c*/Cytochrome b_5

Cytochrome b_5 is a small heme protein (11 kDa) which is responsible for electron-transfer reactions in a number of physiological processes such as reduction of met-hemoglobin and ferric-cytochrome P450.[210] The electron-transfer reaction between ferrocytochrome b_5 and ferricytochrome c has been studied using a number of different techniques.[4,33,211] Perhaps the most extensively studied interprotein electron transfer is that between ferrocytochrome b_5 and ferricytochrome c, and the structural characterization of the cytochrome b_5/cytochrome c complex has been performed largely by NMR spectroscopy.[91]

Kinetic studies allowed the determination of the cross-exchange electron-transfer rates using saturation transfer experiments.[208,209] The intracomplex rate constants were found to be 1.4, 0.5, and 0.7 s^{-1} for reverse electron transfer between unmodified ferricytochrome b_5, the Cys-102-Thr variant, and the Phe-82-Gly, Cys-102-Thr variant, respectively. Consequently, the substitution of Phe-82 with glycine was considered to introduce a negligible effect on the first-order rate of electron transfer, although the invariance of this residue and its simulated dynamic properties have supported a possible role in physiological electron transfer.[212] However, these values seem too low in comparison with the forward rate constant for electron transfer within the preformed complex when an equilibrium calculated from the reduction potentials of the two protein couples is considered, as determined by other methods.[19,213] It was proposed that saturation transfer cannot detect multiple forms of the complex and, if fast-exchange conditions applied, a separate resonance would not be present for this form. Under these conditions, only the slow form of the complex would contribute to the saturation transfer experiment.[213] However, there is no evidence in the NMR spectrum of such a fast exchange behavior. Another possibility for this discrepancy, suggested by Mauk et al.,[33] was the leakage of the magnetic labeling effect to relaxation. It was assumed in the NMR experiment that the paramagnetism of ferricytochrome b_5 does not contribute to the relaxation of the resonances of cytochrome c. Furthermore, a recent NMR investigation of cytochrome b_5/cytochrome c through the analysis of ^{15}N relaxation rates (vide infra)[214] concluded the formation of multiple adducts depending on protein concentration. This may explain the different values observed for electron-transfer rates, since the differ-

ent methods, which were recorded at different concentrations and different ionic strengths, analyze a different situation.

The reorganization energy, λ, for the cytochrome *c*/cytochrome b_5 system was first reported by McLendon and Miller,[215] who measured electron-transfer rate constants in four different complexes. They calculated a λ of approximately of 0.8 V for the sum of the reorganization energies of cytochrome *c* and cytochrome b_5. This reorganization energy can also be calculated from the self-exchange data obtained from NMR data.[35] The λ value of 0.95 V is in very good agreement with the previous value (0.8 V).

Another recent contribution of NMR spectroscopy has come from structural information of the interacting proteins in the complex.[214,216] In the history of this interaction, Salemme[217] first proposed a model of the complex dominated by the complementary charged side chains that surround the exposed hemes in both proteins. Cytochrome b_5 is a negatively charged protein at physiological pH, whereas mitochondrial cytochrome *c* is largely positive. Many experimental studies[33] and theoretical approaches[218−220] were then employed to characterize electron-transfer processes between these two proteins. In these processes, NMR spectroscopy provided evidence of the interaction through chemical shift analysis, first through hyperfine-shifted signals[209,221,222] and then through 2D heteronuclear NMR spectroscopy.[91,202,214,216,223] Titration of labeled cytochrome b_5 in the presence of unlabeled cytochrome *c*[216] and the reverse, titration of labeled cytochrome *c*[214] with unlabeled cytochrome b_5, have been reported. At low protein concentrations and in an equimolecular mixture, two different 1:1 adducts are formed, whereas, at high concentration, a 2:1 adduct is formed.[214]

6.2. Cytochrome *c*/Cytochrome *c* Peroxidase

This system is one of the best studied electron-transfer systems, and there is an X-ray structure of the 1/1 complex of yeast cytochrome *c* peroxidase in association with a cytochrome *c* by Pelletier and Kraut.[9] This general topic was reviewed in 1996 by Hoffman and collaborators.[17] Since the work reported by Gupta and Yonetani showing the spectroscopic evidence for the existence of such complexes,[205] the ^{1}H NMR studies have been mainly performed by Satterlee and colleagues.[224−229] Studies of the interaction of cytochrome *c* peroxidase in the high-spin form[224,225,229] or the low-spin cyanide-ligated form[226,227,229] with various cytochromes *c* (tuna, horse, and yeast) have been undertaken to obtain the stoichiometry of the complexation ($^1/_1$ or $^1/_2$) and the different sites of binding. H/D isotope exchange rates for amide protons[230] and heme hyperfine proton resonances[229] have also been used to study the noncovalent interaction between cytochrome *c* peroxidase and cytochrome *c*. The interface region identified by comparing the H/D exchange rate constants of the free and bound states is similar to the binding interface obtained from the solid-state crystal structure, but a second region located away from the first one was also detected. Thus, NMR experiments support a two-domain binding mechanism at the high concentrations of cytochrome *c* peroxidase and cytochrome *c* employed,[231,232] and now the possibility of secondary sites on this redox enzyme is largely accepted.[44] In a different NMR approach, Worrall and co-workers explained proton and nitrogen chemical shift changes of backbone amides as the result of the interaction between [^{15}N]-labeled yeast-iso-1-cytochrome *c* and unlabeled yeast cytochrome *c* peroxidase.[233]

Another model system for studying possible different site interactions has been the diheme cytochrome *c* peroxidase from *Paracoccus denitrificans* and the complexes that this bacterial enzyme forms with redox partners.[234] It was found by NMR that the peroxidase can accommodate both horse heart cytochrome *c* (a nonphysiological electron donor) and cytochrome c_{550} (the physiological electron donor) in a ternary complex.[234] In contrast, a recent NMR study showed that cytochrome c_{550} and pseudoazurin[235] bind at the same site on the cytochrome *c* peroxidase.[236] Thus, bacterial cytochrome *c* peroxidases, which are distinct in structure and mechanism from eukaryotic cytochrome *c* peroxidases (yeast), afford a parallel model system for investigation.

6.3. Cytochrome c_{552}/Cytochrome Oxidase

There is a computer simulation of the protein−protein interactions between these two proteins which was performed in a docking study.[237] To get experimental information, chemical shift mapping by NMR spectroscopy has also been used to investigate the molecular interaction between two components of the electron-transfer chain from *Paracoccus denitrificans*: the water-soluble fragment of cytochrome c_{552} and the Cu_A domain from cytochrome oxidase.[86] Comparison of [^{15}N,^{1}H]-TROSY spectra of the [^{15}N]-labeled cytochrome c_{552} fragment in the absence and in the presence of the Cu_A fragment showed chemical shift changes mostly located around the exposed heme edge in cytochrome c_{552}.

6.4. Cytochrome *c*/Cytochrome *f*

Structural characterizations of cytochrome *f* have been previously reported, showing unusual features[238,239] such as the sixth ligand, a terminal tyrosine, which coordinates through the α-amino group. Although cytochrome *c* is a nonphysiological partner of cytochrome *f*, the complex of yeast cytochrome *c* and cytochrome *f* from the cyanobacterium *Phormidium laminosum* has been investigated by NMR spectroscopy.[240] Considering the net charges under neutral conditions (−14 for cytochrome *f* and +8 for cytochrome *c*), a strong electrostatic attraction was expected for these two proteins. A combination of NMR spectroscopy and protein docking simulations made it possible to identify two cytochrome *c* binding sites on cytochrome *f*. One site is identical to the binding site previously determined for the natural cytochrome *f* partner plastocyanin (vide infra).[93]

6.5. Cytochrome *c*/Plastocyanin

The rate of reverse electron-transfer reaction within the cytochrome *c*/plastocyanin complex has been determined by NMR line broadening (27 s^{-1} at 298

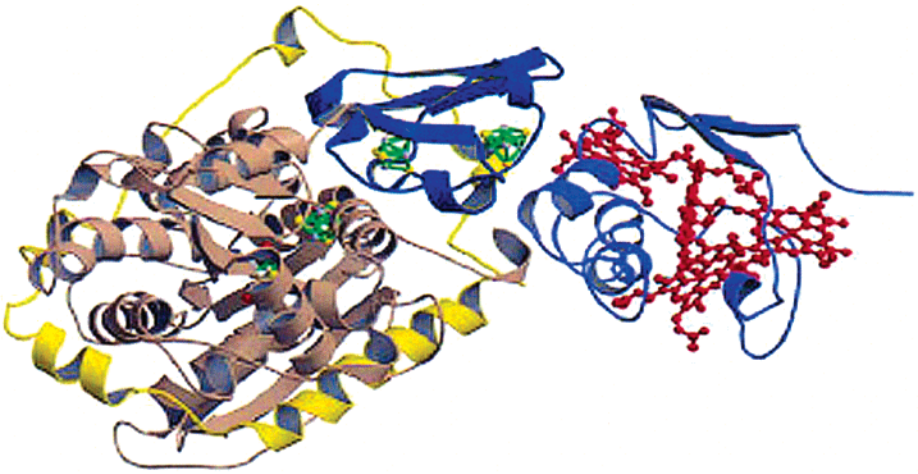

Figure 11. Structural model of a cytochrome c_3−hydrogenase electron-transfer complex using heteronuclear NMR and docking calculations (adapted from ref 251).

K).[18] Later, the complexes of horse ferrous and ferric cytochrome *c* with Cd-substituted pea plastocyanin were characterized by NMR in order to study the binding sites and to study the effects of complex formation.[241]

6.6. Cytochrome c_3–Protein Interactions

The cytochrome c_3 interactions with various electron carriers, mainly ferredoxin,[242−247] flavodoxin,[248,249] and rubredoxin[250] have been studied by NMR due to their possible physiological roles. A recently reported method, using heteronuclear NMR and docking calculations, was used for building structural models of the cytochrome c_3/hydrogenase electron-transfer complex (Figure 11).[251] This model connects the distal cluster of the [Fe]-hydrogenase to heme 4 of the cytochrome. By a similar approach, it has recently been demonstrated that the same heme (heme 4) interacts with cytochrome *Hmc* (see below).[252] The crystal structure of the hexadeca-heme cytochrome *Hmc* has been recently reported.[252] Cytochrome *Hmc* is a high-molecular-weight cytochrome that contains 16 heme groups in a single polypeptide chain. A structural model of a complex with its physiological electron-transfer partner, cytochrome c_3, was obtained by NMR-restrained soft-docking calculations.[252] This method follows a general strategy to study solution complexes between redox partners and has been largely applied by the same authors on other heme protein complexes.[45,253,254] Amino acids of the cytochrome c_3 involved in the interaction with the cytochrome *Hmc* were determined by recording $^1H-^{15}N$ HSQC experiments and analyzing ^1H and ^{15}N chemical shift data. This complex involves the last heme at the C-terminal region of the V-shaped cytochrome *Hmc* and heme 4 of cytochrome c_3.[252]

6.7. Cytochrome *f*/Plastocyanin

The number of protein interactions in the photosynthetic redox chain has been investigated by NMR spectroscopy.[93,204,255] This topic has been recently reviewed.[204] Thus, the interaction of soluble forms of cytochrome *f* with plastocyanin from different origins has been largely studied by NMR. The structure of a transient complex of cytochrome *f* and plastocyanin has been predicted from shifts in the spectrum of plastocyanin on binding cytochrome *f*.[92] Diamagnetic chemical shift changes and intermolecular pseudocontact shifts in the NMR spectrum were used as input in restrained rigid-body molecular dynamics calculations.

The interaction of parsley plastocyanin with turnip cytochrome *f* has also been investigated by NMR spectroscopy together with protein docking simulation.[256] The results of NMR studies indicate that this plastocyanin and its spinach adopt different orientations in their complexes with cytochrome *f*. As an example, in contrast to other electron-transfer complexes,[92] the plastocyanin/cytochrome *f* complex from *Phormidium laminosum* is predominantly controlled by hydrophobic interactions.[93] The influence of hydrophobic patch mutations has been studied by NMR spectroscopy using titration and chemical-shift mapping.[257]

6.8. Cytochrome c_6/Cytochrome *f*

In certain cyanobacteria, electron transport between the membrane-bound complexes cytochrome *bf* and photosystem **I** is fulfilled by cytochrome c_6. To address the question of molecular recognition in cytochrome c_6, Ubbink and collaborators[258] have investigated complex formation with cytochrome *f* using heteronuclear NMR. Chemical-shift perturbation analysis reveals a binding site on *Anabaena* cytochrome c_6 which consists of a predominantly hydrophobic patch surrounding the heme substituent methyl 5. Such a hydrophobic site is similar to that proposed previously for the interaction of cytochrome c_6 with photosystem **I**.[259] Since it was found that there is no evidence for specific complex formation with a different acidic cytochrome c_6 from *Synechococcus* under similar experimental conditions, it was concluded that different organisms utilize distinct mechanisms of photosynthetic intermolecular electron transfer.[258]

6.9. Cytochrome b_5/Hemoglobin (Myoglobin)

The study of the interaction between cytochrome b_5 and hemoglobin is important because it allows the

possibility to investigate biological electron transfer between physiological partners.[210,260] Because of its physiological importance, the system has been largely investigated by different groups.[15,261−263] In particular, electron-transfer rates were determined using heme proteins reconstituted with photoactive heme groups.[264,265] The kinetics of met-hemoglobin reduction by cytochrome b_5 have also been studied by saturation transfer NMR and stopped-flow NMR.[266] A forward rate constant $k_f = 2.44 \times 10^4$ M^{-1} s^{-1} has been measured from the stopped-flow experiment, and a reverse rate constant $k_b = 540$ M^{-1} s^{-1} has been calculated at pH 6.2 and 298 K from a saturation transfer NMR experiment. As previously reported (see section 2), this technique is very useful for the measurement of kinetics in the uphill direction. Since the rate constant directly determined from the NMR experiment is a combination of self-exchange between cytochrome b_5 (II) and cytochrome b_5 (III) and cross-exchange between cytochrome b_5 (III) and deoxyhemoglobin, it is necessary to measure the self-exchange rate independently. It was found that the maximum rate for electron transfer lies at the lowest ionic strength, according to a bimolecular collisional pathway. Under these physiologically relevant conditions, it seems that a specific protein−protein interaction, as suggested from model[267] and hemoglobin variant studies,[262] may not be necessary for this electron-transfer reaction.

The interaction between cytochrome b_5 and myoglobin is weak but could be a physiological one.[210] Thus, experimental NMR methods and theory have also been used to study this interaction and electron transfer between horse heart Mb and trypsin-digested bovine cytochrome b_5.[268] The paramagnetic hyperfine shifts in the cytochrome b_5 ^{1}H NMR spectrum are perturbed by met-myoglobin, indicating the formation of a specific complex with a 1/1 stoichiometry.[269] The binding affinity of met-myoglobin for cytochrome b_5 has been measured by ^{1}H NMR titrations, giving $K_a = 1 \times 10^3$ M^{-1} at pH 6.0.[268] Isothermal titration calorimetry and 2D NMR methods have also been used to independently investigate the effect of charge neutralization on myoglobin/cytochrome b_5 binding. It was found that the neutralization of the heme propionates of Zn myoglobin increases the second-order rate constant of the electron transfer by as much as 100-fold, depending on pH and ionic strength.[270]

Recently, the transient complex of bovine myoglobin and cytochrome b_5 has been investigated using a combination of NMR chemical shift mapping, ^{15}N relaxation data, and protein docking simulations.[206] The data support the emerging view that this complex consists of a dynamic ensemble of orientations in which each protein diffuses over the surface of the other.

6.10. Cytochrome P450/Putidaredoxin

Cytochrome P450cam from *Pseudomonas putida* catalyzes the regio- and stereospecific hydroxylation of D-camphor at the 5-exo position by utilizing two reducing equivalents and a molecular oxygen.[271] Thus, the reaction cycle requires two electrons which

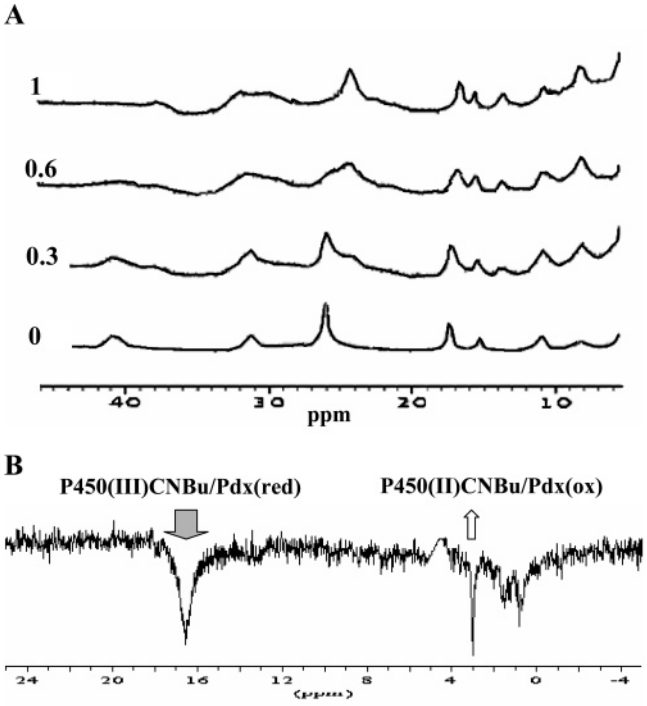

Figure 12. (A) ^{1}NMR spectra using PASE of the cytochrome P450cam(^{n}BuNC) complex in 10 mM potassium phosphate D_2O buffer, pD 7, at 298 K with no putidaredoxin and with 0.3, 0.6, and 1 equiv of putidaredoxin. (B) ^{1}NMR difference spectrum obtained by magnetization transfer of the 1-methyl of the heme of cytochrome P450 inside the diprotein complex cytochrome P450cam−putidaredoxin (adapted from ref 83).

are sequentially transferred, via a shuttle mechanism, by an iron−sulfur protein, putidaredoxin, between cytochrome P450cam and putidaredoxin reductase. A number of studies have been conducted to elucidate the mechanism of the electron-transfer reaction, which is essential for the catalytic cycle.[272−278] Despite this accumulation of results, the structural factors regulating the electron-transfer reaction still remain to be better defined, in particular the specific recognition between cytochrome P450cam and the redox partner, putidaredoxin. The biophysical characterization of cytochrome P450cam has been performed mostly using EPR,[279] infrared spectroscopy,[280,281] and electronic spectroscopies.[282] In contrast, only a few NMR studies have been described and the ^{1}H NMR spectra are characterized by broad signals in both the reduced and oxidized states.[283−287] However, the chemical shifts of the cysteinate fifth ligand protons and some of the heme protons have been assigned through NOESY spectra in the diamagnetic cytochrome P450Fe(II)CO[288] and, recently, in the low-spin and high-spin ferric forms.[79]

The first kinetic investigation by ^{1}H NMR of the electron transfer between cytochrome P450 and putidaredoxin was reported in 1999.[83] Despite its relatively large molecular weight, the diprotein complex formation is detectable (Figure 12). Isocyanide was bound to cytochrome P450cam[289] in order to increase the stability of the protein in both the reduced and the oxidized state. Actually, the crystal structures of the ferric and ferrous *n*-butyl isocyanide complexes of cytochrome P450 have recently been determined.[290]

Cytochrome P450 is physiologically reduced by putidaredoxin, and the presence of a redox equilibrium between the two proteins is well-known. At high concentration (0.8 mM for ^{1}H NMR), the complex is quite stable, since the K_d is 10−88 mM between cytochrome P450 (red) and putidaredoxin (ox).[291] To determine the intra- or intermolecular nature of the electron transfer, the dilution effect on the relaxation of the proton of the proximal cysteine was measured in the reduced form. The absence of variation of the observed T_1 with dilution was indicative of a lack of any contribution of the intermolecular mechanism.[83] Assuming an intramolecular mechanism, the rate constant was estimated using magnetization transfer experiments (Figure 12).[83] In this study, it was necessary to perform the saturation of cytochrome P450 signals in the paramagnetic state and measure the variation of the intensity of the corresponding proton in the reduced diamagnetic form. This permits the accumulation of transferred saturation in the ferrous form although the relaxation rate of the oxidized signal is fast compared to the electron-transfer rate. A rate constant of 27 s^{-1} was found.[83] Previously reported values for cytochrome P450/putidaredoxin systems were 17.5 s^{-1} and 160 s^{-1}.[292]

Actually, bacterial cytochrome P450cam and putidaredoxin are one of the rare redox pairs for which each three-dimensional structure is now available at the atomic level.[293−296] Various spectroscopic studies including EPR,[279,297] IR,[281] and resonance Raman[297] have also been carried out to examine the interaction between cytochrome P450cam and putidaredoxin. To investigate the specific interaction between putidaredoxin and cytochrome P450cam from the structural point of view,[298] NMR is also a powerful tool, even though the analysis of such a large complex is not very easy.

First, in the diamagnetic ferrous-CO form of cytochrome P450cam, the ring current of the porphyrin separates several resonances of the protons near the heme from the crowded signal region between 0 and 10 ppm.[288] Thus, it was possible to assign the cysteinate fifth ligand protons and several of the heme protons through 1D and 2D spectra at 500 MHz. This assignment was very useful to study the structural modification observed after putidaredoxin interaction (vide infra). ^{13}CO NMR chemical shifts have also been measured in the CO form, showing the steric and electrostatic interactions acting on the CO ligand are influenced by the substrate.[299]

A multidimensional NMR study on the complex showed that the binding of putidaredoxin structurally perturbs the several regions involving the substrate access channel.[300] Later, Morishima and collaborators[301] assigned other signals from the substrate, D-camphor, and Thr 252 by two-dimensional NOESY and one-dimensional NMR spectra of the free substrate using wild type and the Thr-252 mutant of cytochrome P450cam. From these new results, it was suggested that the binding tilts the heme plane, leading to the movement of D-camphor and the axial cysteine to the heme iron by 0.1−0.5 Å. It was also proposed from NMR studies that putidaredoxin-induced specific conformational changes of cytochrome P450cam would facilitate the electron transfer to oxy-cytochrome P450cam.[302] Recently, to obtain a more detailed picture of the interactions between cytochrome P450cam and putidaredoxin, Pochapsky et al.[300] have applied multidimensional NMR methods to the characterization of the cytochrome P450cam/putidaredoxin complex. Using new resonance assignments, these authors propose a model in which the primary binding site of putidaredoxin, at the proximal face of cytochrome P450cam, is not the same one that results in electron-transfer activity. It was suggested that the specific binding is not a passive event but a delicate balance of structural and dynamic factors, related to the presence of the substrate.[300]

7. Conclusion

NMR represents a powerful method for studying the dynamic processes involving electron transfer from both the kinetic and structural points of view. The research reviewed in this article clearly demonstrates that it is now possible to obtain a wealth of information from the 1D and 2D NMR experiments even in the paramagnetic state. For example, the rate of electron transfer self-exchange reactions can be determined directly by NMR experiments over a large range of rates. This property is especially interesting for the determination of the rate at infinite ionic strength, which opens the door to the reorganization energy calculation. Heme proteins often display charged residues at the surface, and any variation of the ionic strength is able to induce dramatic variations of the rate in the cases of both self-exchange and intermolecular reactions. This is also one of the best methods because it is not necessary to know either the one-electron redox potential of the complex or the rate constants for self-exchange in any partner complex, as would be the case if cross-reactions and the Marcus theory were used to estimate the rate constant. In the latter possibility, the theory proceeds from the assumption that, within the activated complex, the work terms for the self-exchange and cross-reactions are the same,[56] which is not always the case, in particular when the electron-transfer partners present different charges on the protein surface.

The kinetics of electron transfer are determined by NMR methods when the system is under equilibrium. Generally, it is possible to measure by other methods the rate of electron transfer under thermodynamically favorable conditions (downhill direction). However, the kinetic study of an electron-transfer reaction will never be complete without knowing the reaction rate in the backward direction, which is thermodynamically unfavorable. This is difficult, since the reaction cannot proceed spontaneously in that direction. The use of NMR methods (through saturation transfer or determination of the relaxation time) overcomes this difficulty in intermolecular,[266] multicenter,[164] and intracomplex[83] metalloprotein electron transfer, as long as the dynamic of the equilibrium is large enough.

NMR is also very well suited to the study of especially weak protein−protein interactions, as no crystallization is required. Most of these methods are

now routinely applied for complexes with total mass as large as 100 kDa.[39] As recently suggested,[38] and partly demonstrated in section 4.2, one the most exciting immediate prospects is structure determination of small membrane proteins reconstituted in lipid micelles involved in an electron-transfer process.

In conclusion, NMR has now been applied to a wide range of intraprotein and interprotein electron-transfer reactions and is rapidly becoming an essential tool in this research area.

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