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Proton nuclear magnetic resonance study of the binary complex of cytochrome P450cam and putidaredoxin: interaction and electron transfer rate analysis

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Abstract **A ^{1}H nuclear magnetic resonance study of the complex of cytochrome P450cam-putidaredoxin has been performed. Isocyanide is bound to cytochrome P450cam in order to increase the stability of the protein both in the reduced and the oxidized state. Diprotein complex formation was detected through variation of the heme methyl proton resonances which have been assigned in the two redox states. The electron transfer rate at equilibrium was determinated by magnetization transfer experiments. The observed rate of oxidation of reduced cytochrome P450 by the oxidized putidaredoxin is 27 (± 7) per s.**

Key words: Cytochrome P450; Putidaredoxin; Electron transfer; Nuclear magnetic resonance; Isocyanide

1. Introduction

Electron transfers between proteins have been shown to play an important role in biological systems. Various approaches are used to study the different factors controlling the electron transfer between the donor and acceptor sites. Since many electron transfer reactions occur inside protein complexes, analysis of a well-defined diprotein complex, constituted of the donating and the receiving species, is of particular interest for the knowledge of protein-protein interactions and electron transfer pathways. However, a few examples of such diprotein complexes have been reported. An important example is the cytochrome P450cam-putidaredoxin (cyt P450-Pdx) interaction which is currently under intensive studies [1–8]. Cyt P450cam is a bacterial soluble form of cyt P450, which catalyzes the hydroxylation of camphor [9]. The reaction cycle requires two electrons which are sequentially transferred by an iron-sulfur protein, Pdx, between cyt P450 and Pdx reductase. Both the complex formation and the electron transfer of wild-type and mutated forms of these proteins have been studied using a variety of spectroscopic modalities. Although nuclear magnetic resonance (NMR) is a sensitive technique for the study of protein interactions, analysis of such a large complex is difficult. However, the ^{15}N NMR shift of ^{15}N-labelled cyanide complex of cyt P450 in the presence of Pdx has been studied [10]. It should be noted that Pdx has been extensively studied by NMR. The solution structure [11] as well as a model of the cyt P450-Pdx interaction [12] was proposed by Pochapsky et al. Further, ^{15}N measurements of ^{15}N-labelled Pdx were performed with and without cyt P450 [12,13].

NMR is a powerful spectroscopic method for reaction rate determination over a large range of chemical exchange kinetics. In the case of a moderately rapid reaction, the magnetization transfer experiment is the most widely applied method [14]. Electron transfer under thermodynamic control can be considered as a chemical exchange between two redox states. Thus, the NMR rate determination of self-exchange reactions was first performed on the mixture of ferri-ferro-cyt *c* [15]. Later, magnetization transfer has successfully been applied to several metalloproteins with the aim of resonance assignment in one redox state from resonance assignment in the other state. The electron transfer rate determination in other heme proteins has also been extensively studied both by magnetization transfer or relaxation measurements [16]. Heme proteins are particularly suitable because 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.

In this report, we present NMR experiments on alkyl isocyanide adducts of cyt P450. These ligands give stable complexes with cyt P450 on ferric and ferrous states. Consequently, we are expecting a weak energy of reorganization between the two redox states of cyt P450 (RNC), which is in favor of electron exchange. This was previously demonstrated on myoglobin [17] and hemoglobin [18]. Heme resonances of the isocyanide adduct of the ferrous form are assigned by analogy with previous assignments obtained with the carbon monoxide adduct of reduced cyt P450 [19]. Addition of an equimolar amount of oxidized Pdx in a solution of oxidized cyt P450 (RNC) induces the formation of a stable diprotein complex, as detected by ^{1}H NMR. Partial reduction of the binary complex allows us to detect electron transfer by magnetization transfer experiments. By these experiments, three heme methyl resonances have been assigned in the *n*-butyl isocyanide low spin complex of oxidized cyt P450. Because the corresponding resonances of the heme methyls of the reduced form are under the very intense diamagnetic

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region of the complex (58 kDa), no classical analysis of the magnetization transfer experiment was available. Consequently, we used an new treatment of the results obtained through the spectrum difference mode.

2. Materials and methods

Cyt P450cam (CYP101) was expressed in *Escherichia coli* TB1 cells and purified to an absorption ratio 392 nm/280 nm of 1.3 as previously described [20]. Camphor was removed by dialysis against 100 mM Tris-HCl buffer at pH 7 and a passage through a Sephadex G25 column equilibrated with the same buffer. The eluted substrate free P450cam was checked on a UV-visible spectrometer (Uvikon 941) and then dialyzed against 100 mM D_2O phosphate buffer at pD 7 and concentrated by ultrafiltration to a final concentration of about 0.8 mM. Protein solutions were flushed with argon before being transferred to NMR tubes which were previously flushed with argon. The transfer is preceeded by an addition up to 10 mM of *n*-butyl isocyanide in a methanolic solution for the isocyanide complex. Reduction is accomplished by addition of a small amount of a previously degassed solution of sodium dithionite.

Pdx was expressed in *E. coli* strain TB-1 [21]. The competent cells were a generous gift of Prof. S.G. Sligar (University of Illinois in Urbana-Champaign). Purification was performed according to a slightly modified version of published procedures [22].

n-butyl isocyanide was obtained from Aldrich. ^{1}H NMR spectra are recorded on a Bruker DMX 500 spectrometer at 500.13 MHz with a 5 mm inverse ^{13}C-^{1}H probe. The one dimensional (1D) spectra of oxidized forms were acquired with a sweep width of 40 kHz along with a 90° pulse preceeded by the PASE method [23]. 1D and NOESY spectra of the reduced form were acquired as previously described [19]. Proton chemical shifts were measured relative to HDO and were expressed relative to TSP through the residual water signal.

3. Results

3.1. Cyt P450-n-butyl isocyanide complexes

Addition of *n*-butyl isocyanide to substrate free ferrous cyt P450cam results in a complex showing a red-shifted Soret band at 455 nm. Whereas isocyanide binding to microsomal cyt P450s induced a double Soret band [24], a single Soret band is observed with cyt P450cam as previously reported with ethyl isocyanide [25]. NMR spectra of a good quality can be obtained even at 313 K since the isocyanide adduct is stable at this temperature. By analogy with the previous studies realized on the carbon monoxide adduct [19], a NOESY map (not shown) permits the assignment of meso and the methyl protons of the porphyrin. The chemical shifts of the heme methyl groups are given in Table 1. The ^{1}H NMR spectrum of the adduct of *n*-butyl isocyanide to substrate free ferric cytochrome P450cam is shown in Fig. 1A. The spectrum is characteristic of a low spin complex of cyt P450 [26,27]. Due to a long electronic spin relaxation time in the low spin ferric state, very short relaxation times (<10 ms) are observed for the hyperfine shifted signals. Thus, direct assignment of the heme methyl signals cannot be obtained. Any

Table 1
Heme methyl assignments (ppm) for various forms of cyt P450cam

	Fe^{III}-nBuNC-Pdx low spin	Fe^{III}-nBuNC low spin		Fe^{II}-nBuNC s = 0
	298 K	298 K	313 K	313 K
5-CH_3	24.5	25.9	24.9	3.37
1-CH_3	16.7	17.1	16.8	3.18
8-CH_3	-	-	-	3.38
3-CH_3	−1.0	−1.5	−1.7	3.48

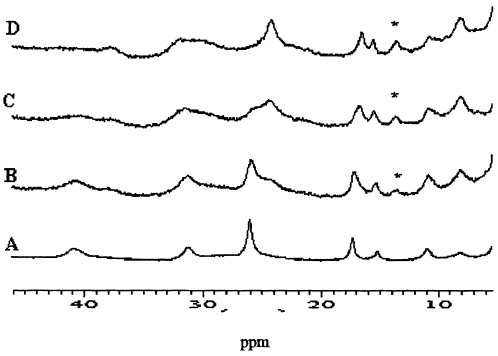

Fig. 1. ^{1}H NMR using PASE [23] of the cyt P450cam (nBuNC) complex in 10 mM potassium phosphate D_2O buffer, pD 7 at 298 K. A: no Pdx, B: 0.3, C: 0.6, D: 1 equivalent of Pdx, respectively.

detection of self-exchange electron transfer by magnetization transfer experiments on a partially reduced sample of cyt P450 (nBuNC) failed. If any electron transfer occurs, the rate is too slow to be detected by NMR. Fortunately, assignment was successful using saturation transfer experiments after addition of Pdx (see below).

3.2. Cyt P450-Pdx interaction

Fig. 1 shows the ^{1}H 1D-PASE NMR spectrum of oxidized cyt P450cam (nBuNC) in the presence of various amounts of Pdx. The addition of Pdx induces a shift of the hyperfine shifted signals of the heme methyl groups. All the shifted resonances move to the high field which should correspond to a decrease of the electron spin density on the heme ring or a change of the magnetic anisotropy. The relative contribution of these effects is unclear because Pdx binding can induce structural modifications on the proximal ligand and/or the isocyanide ligand. Similar conclusions were previously proposed from the ^{15}N NMR study of the cyanide adduct of cyt P450cam [10]. In the case of unligated but camphor bound cyt P450cam, the interaction with Pdx was shown by resonance Raman to shift the spin state equilibrium of cyt P450cam to the low spin state [28]. With an isocyanide-ligated cyt P450cam, the observed shifts for all the paramagnetic resonances are more suggestive of electronic modification inside the heme pocket rather than a specific sterical heme constraint induced by the interaction with Pdx. Also noticeable in Fig. 2 is the presence of the $N_{\delta 1}H$ of His-49 localized in the C-terminal cluster of the Pdx [11]. This exchangeable proton remains present even in D_2O. Unfortunately, no data on the H-D exchange were previously reported [29]. Thus, any relationship between diprotein complex formation and the kinetics of the isotopic exchange is precluded for the present time.

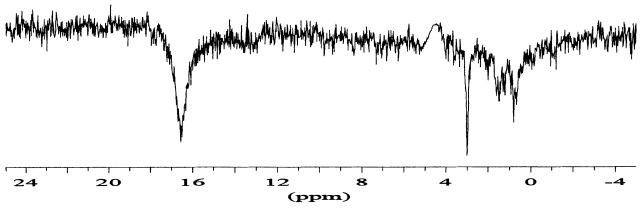

Fig. 2. ^{1}H NMR difference spectrum obtained by irradiation of the 1-Me at 16.7 ppm of the heme of cyt P450 inside the diprotein complex cyt P450cam (nBuNC)-Pdx (1/1) in 10 mM potassium phosphate D_2O buffer, pD 7 at 298 K.

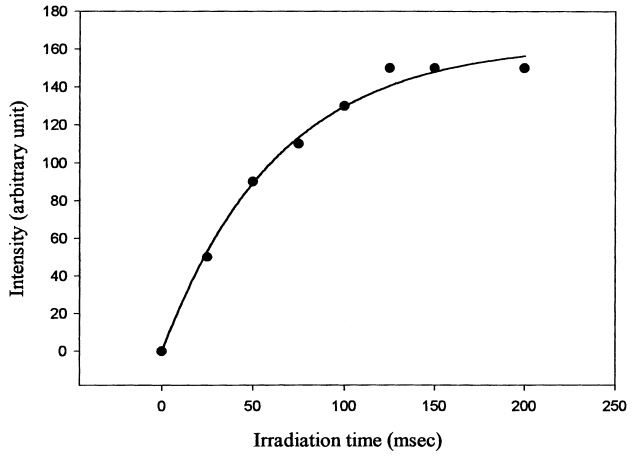

Fig. 3. Plot of the intensity of the 5-Me signal in the FeII form (magnetization transfer (M_0^{-M}t)) as a function of the irradiation time. A: experimental, B: fitted data.

3.3. Detection of electron transfer reaction

The diprotein complex was approximately half-reduced with sodium dithionite. Addition of sodium dithionite to oxidized cyt P450 (nBuNC) caused a visible spectral change, i.e. an increase of absorbance at 455 nm with a concomitant decrease at 425 nm. The percentage of reduced versus oxidized forms of cyt P450 (nBuNC) was based on the molar absorption of each form. The proton NMR spectrum displays broad signals, between 10 and 30 ppm, corresponding to heme resonances of the low spin ferric form and the β-proton of the proximal cysteine of the reduced form at −2.63 ppm (not shown). This proton was assigned by comparison with cyt P450-CO [19]. The paramagnetic signals were detected using the PASE sequence which improved the quality of the baseline [23], whereas the reduced signals are better detected with a classical sequence of presaturation of the residual water. Consequently, direct integration of the oxidized and reduced signals cannot be accomplished and the estimate of each redox species of the cyt P450 (nBuNC) was obtained by the ratio of the two Soret bands at 455 and 428 nm, as previously indicated. Since there is no direct self-exchange reaction in the partially reduced cyt P450 (nBuNC), any magnetization transfer should correspond to an electron exchange between Pdx and cyt P450. Fig. 2 displays the results of the difference spectra obtained by irradiation of the 1-heme methyl signal in the oxidized form. Efficient magnetization transfers are observed between the two redox states. Based on the previously assigned heme methyl resonances in the diamagnetic state, these magnetization transfers allow us to assign the ferric protein signals (Table 1).

3.4. Kinetic determination of the electron rate transfer

In order to determine the intra- or intermolecular nature of the electron transfer, we measured the effect of dilution by addition of degassed buffer inside the NMR sample of a partially reduced diprotein complex. In difference spectroscopy, quantitation without standardization of signals from different samples is imprecise. Therefore, we measure the dilution effect on the relaxation time of the β-proton of the proximal cysteine of cyt P450 (nBuNC) in the reduced form (−2.63 ppm). This observed T_1 is multicomponent and includes the T_1 of the cysteine proton in the fully reduced diprotein complex and the effective T_1 of the cysteine proton in a mixed complex under electron exchange. This prevents the precise calculation of the rate transfer by the method previously used for the study of a different diprotein complex formed with the association of cyt *c* and cyt *b*5 [30]. However, the absence of variation of the observed T_1 with dilution is indicative of a lack of any significant contribution of the intermolecular mechanism.

Assuming an intramolecular electron transfer mechanism, the magnetization transfer experiments could permit to estimate the rate constant. The classical Bloch equation for one site in the two site exchange network is reported below [14]:

$$\mathrm{A(cyt\ P450FeII - Pdx\ ox)} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{B(cyt\ P450FeIII - Pdx\ red)}$$

$$\frac{\mathrm{d}(M_Z^A)}{\mathrm{d}t} = -\frac{M_Z^A - M_0^A}{T_1^A} - \frac{M_Z^A}{\tau_A} + \frac{M_Z^B}{\tau_B}$$

where

$$\tau_A = \frac{1}{k_1}$$

and

$$\tau_A = \frac{1}{k_{-1}}$$

Measurement of the fractional change in the intensity of A after full saturation of B permits to calculate the rate constant k_1, provided that T_1^A is known. An improvement of this calculation was recently proposed [31] which permits more precise values of k_1 to be obtained even when the measurements of T_1^A and M_0^A are not known. In the present study, it is necessary to perform the saturation of cyt 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 (under the control of the T_1^{Red} and k_1) in spite of the fact that the relaxation rate of the oxidized signal ($R_1^{Ox} = 1/T_1^{Ox}$) is fast compared to the electron transfer rate. Consequently, because the heme methyl signals of the reduced form are under the crowded region around 3 ppm, we are unable to measure either their intensity without irradiation of the ferric signal or their relaxation time.

The variation of the reduced signal intensity (in the difference mode) after time-dependent saturation transfer and the calculated curve are displayed in Fig. 3. The curve was fitted using the following equation in order to get T_1^{App} and $(M_0 - M_\infty)$.

$$\frac{M_0 - M_t}{M_0 - M_\infty} = (1 - e^{(t/T_1^{App})})$$

where M_∞ denotes the observed magnetization after a long-time saturation.

Thus, T_1^{App}, the apparent relaxation time of the reduced signal, can be obtained without knowledge of M_0 and has been found to be 36 (±10) ms. Finally, the cyt P450 oxidation rate constant, k_1, can be expressed as follows:

$$\frac{1}{T_1^{App}} = \frac{1}{T_1^{Red}} + k_1 \text{ so } k_1 = \frac{T_1^{Red} - T_1^{App}}{T_1^{Red} * T_1^{App}}$$

where T_1^{Red} is the T_1 of the reduced form without exchange.

As previously noted, no exact determination of the relaxation time of the heme methyls in the reduced form can be performed. However, an estimate of a range of possible values can be formulated based on the data available on various hemeproteins. A large range of T_1, i.e. from 0.5 to 2 s, results in a very weak change in the electron rate constant, from 25.8 to 27.3 s^{-1}. This weak dependence of the T_1 values is consistent with a small relaxation rate compared to the electron transfer rate. Thus, the rate constant of cyt P450 oxidation by oxidized Pdx is found to be 27 ($\pm$7) s^{-1}.

4. Discussion

The assignment of the heme methyl resonances of cyt P450 (nBuNC) was performed on both the diamagnetic ferrous form and the ferric low spin form. The reduced form was assigned at 313 K which permits a significant improvement in the quality of the spectra and allows us to use classical correlation NMR methods. The assignment of the ferric low spin form of the nBuNC complex of cyt P450 was obtained through magnetization transfer experiments. Further assignments of various paramagnetic states of cyt P450 are now accessible and will be reported elsewhere. It should be underlined that previous NMR studies have also been performed on the structural interactions between cyt p450 and Pdx. In particular ^{15}N-^{1}H HSQC spectra were acquired using ^{15}N-labelled Pdx [11,13]. These experiments were conducted in a cyt P450/Pdx ratio of 3/1 under fast exchange conditions for Pdx (free and complexed), the line width of most of the signals being too broad to get good correlation spectra in equimolar conditions [13]. In contrast, the proton NMR results presented herein were obtained in an equimolar ratio and at a low salt concentration which permits detection of the variation of cyt P450 resonances induced by diprotein complex formation.

Cyt P450cam is physiologically reduced by Pdx and the presence of a redox equilibrium between the two proteins is well known [1,2,32]. The ratio of the reduced versus oxidized states inside the diprotein complex is related to the difference of the redox potential between these two proteins. The determination of the percentage is difficult since large variations of the redox potentials have been found, depending on whether the proteins are free or associated. Further, the equilibrium constant of the complex is also dependent on the redox state of the proteins. These parameters are very important when the studies are performed in dilute conditions. At higher concentrations ($\sim$0.8 mM, for NMR experiments), the complex is quite stable since the K_d is 10–88 μM between cyt P450 (red) and Pdx (ox) [2,5]. The redox potential values for the diprotein complex are −303 mV (substrate free) and −173 mV for cyt P450 and −196 mV for Pdx, respectively [32]. The corresponding values when the isocyanide is bound have not been determinated, but a similar shift of the redox potential to more positive values may be expected. An increase of the oxidation-reduction potential has been previously suggested in the case of the phenyl isocyanide adduct of cyt P450scc [33]. Because of the small difference between the redox potentials of the two proteins, some reduction of the Pdx should also occur. The partial reduction of the diprotein complex results in the formation of the mixed cyt P450 (ox)-Pdx (red) complex, but to some extent fully reduced or fully oxidized complexes should also be present in solution. First, it should also be noted that (i) self-exchange reactions have shown to be very slow for Pdx [11] and undetectable by NMR for cyt P450 (this work) and (ii) the intermolecular electron exchange was found to be negligible by measuring the effect of dilution with the diprotein complex. Second, one of the great advantages of the magnetization transfer experiment is the ability to focus on the mixed complex with reduced Pdx by irradiation of a ferric signal of cyt P450. Thus, even in the presence of a complex which is inactive in terms of electron transfer, i.e. fully reduced or fully oxidized, the magnetization transfer efficiency is only related to the intra-complex electron transfer rate. The present analysis of data obtained by ^{1}H NMR difference spectroscopy permits determination of the rate constant for the oxidation of isocyanide bound ferrous cyt P450 by oxidized Pdx. A rate constant of 27 ($\pm$7) s^{-1} was found. Previously reported values for the complex cyt P450 (camphor bound)-Pdx were 17.5 s^{-1} [1] and 160 s^{-1} [5]. Finally, we note that the electron transfer rate from reduced Pdx to cyt P450 cannot be directly calculated without a precise knowledge of all the redox potentials in the presence of isocyanide. Since the spectrum does not give the exact proportion of the redox states of the diprotein complex, additional experiments are necessary to determine this electron transfer rate. Further work will also focus on the assignment of NMR resonances which could permit assessment of the redox state of both proteins inside this diprotein complex.

5. Conclusion

This work constitutes the first kinetic investigation by NMR of the electron transfer between cyt P450 and Pdx. In spite of its relatively large molecular weight, the diprotein complex formation is detectable. Magnetization transfer experiments can be performed which permit both the assignment of the heme methyls of the ferric cyt P450 (nBuNC) and the electron rate constant determination between reduced cyt P450 and oxidized Pdx. Thus, NMR spectroscopy can be used not only for the characterization of structural modifications but also for efficiently determining kinetic parameters over a large range of rates. Such an approach can be helpful to refine the cyt P450-Pdx model both from the structural and functional point of view.

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