^{1}H-NMR study of diamagnetic cytochrome P450cam: assignment of heme resonances and substrate dependance of one cysteinate β proton

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Received 17 April 1997; revised version received 18 July 1997

Abstract The ^{1}H-NMR study of diamagnetic cytochrome P450cam Fe^{II}-CO has been performed for the first time. Chemical shifts of the cysteinate fifth ligand protons and of several heme protons have been assigned through 1- and 2-dimensional spectra at 500 MHz. A substrate dependance has been observed for the resonance of the cysteinate proton detected in the high-field region.

Key words: Cytochrome P450; ^{1}H-NMR; Substrate interaction

1. Introduction

^{1}H-NMR is a powerful method, especially for the study of hemoproteins which present large variations of chemical shifts in the paramagnetic and diamagnetic oxidation states. In the case of cytochrome (cyt.) P450cam, a bacterial soluble form of cyt. P450, the first proton NMR spectrum of the paramagnetic form has been reported in 1972 [1]. While many studies have been devoted to the NMR analysis of various other paramagnetic hemoproteins such as myoglobin, hemoglobin and cytochrome *c* [2–4], the use of ^{1}H-NMR in the case of cyt. P450s was focused during many years on the T_1 relaxation measurements of water and various substrate protons [5–7]. That the number of studies is relatively few [8–10] is related to the presence of the long electronic spin relaxation time which leads to large linewidths of the paramagnetic proton signals even in the low spin state of the iron [5]. However, recently the first assignments of several resonances of the heme have been performed in both low and high spin forms of ferric enzymes [11–13]. Concerning the diamagnetic form, although the large ring current shift of the porphyrin is expected to displace several resonances outside the very crowded 0–10 ppm region, no ^{1}H-NMR spectra have been reported. We recently published the ^{13}C-NMR spectra of the ^{13}C-labelled carbon monoxide (CO) adduct of the ferrous cyt. P450cam [14]. We present herein the assignment of the cysteinate protons of the heme resonances for the diamagnetic cyt. P450cam. The cysteinate proton detected in the high-field region was found to be dependant on the nature of the substrate bound to cyt. P450cam.

2. Materials and methods

Cytochrome P450cam (CYP101) was expressed in *Escherichia coli* and purified to a 392:280 nm absorbance ratio greater than 1.2 as previously described [14,15]. Cytochrome P450lin (CYP111) was a gift from Prof. I.C. Gunsalus (University of Illinois, Urbana-Champaign, IL). It was purified to a 392:280 nm absorbance ratio of 1.5 [16]. Exchange with deuterated buffer (100 mM potassium phosphate, 4% saturated substrate ((1R)-camphor or linalool) in D_2O, pD 7) and concentration were performed by ultrafiltration. Substrates, as well as preparation of the reduced samples [14], were obtained as previously reported. UV-visible spectra were recorded on an Uvikon 941 spectrometer.

^{1}H-NMR spectra were recorded on a Bruker DMX 500 spectrometer at 500.13 MHz with a 5 mm reverse ^{13}C-^{1}H probe. A sweep width of 9 kHz was employed along with a 90° pulse and a repetition time of 1.2 s. Typical NOE difference spectra were obtained by the difference of a total of 7 K scans with interleaved on- and off- resonance irradiation (between 25 and 200 ms) [17]. Residual water signal was suppressed by presaturation. For clarity, the FID was filtered during the processing over 0.5 ppm around the water signal using Bruker procedure. 2D-NOESY spectrum was acquired using standard pulse sequence; quadrature detection was performed using TPPI. The mixing time was set to 150 ms, phase-shifted sine bell functions were applied in both dimensions. 2D-COSY spectra were acquired both in magnitude- and phase-sensitive modes. Proton chemical shifts were measured relative to HDO and were expressed relative to TSP.

Representation of the heme pocket, as well as determination of some interproton distances, was performed using the software SYBYL (Tripos, Inc.) on an Indigo 2 machine (Silicon Graphics). The atomic coordinates of the ternary structure camphor-bound cyt. P450cam–CO [18] were obtained at the Brookhaven Data Bank with internal reference 3CPP.

3. Results

The full spectrum of the cyt. P450cam Fe^{II}–CO in the presence of d-camphor, at 313 K, is presented in the Fig. 1, with expansions of the low- and high-field parts. Fig. 2 shows the temperature influence on these regions of the spectrum. The remarkable improvement in the quality of the spectra, obtained by increasing temperature, is mainly associated with a drastic decrease of the line width of the signals. Signal narrowing permits us to use classical NMR methods for proton assignment, such as 1D-NOE-difference or 2D-NOESY experiments.

Because the cysteinate protons are near the porphyrin ring, we may expect an important shielding of these proton resonances. Inspecting the high-field region of the spectrum in Fig. 1, we can detect a signal at −2.67 ppm which seems to be a good candidate for the resonance of a cysteinate proton. Fig. 3a shows the NOE-difference spectrum obtained upon pre-irradiation of the resonance at −2.67 ppm with a pulse of 50 ms duration. Two large NOEs are detected at lower field. The spectrum displays a sharp peak at −0.52 ppm and a broader one at −0.62 ppm, which should correspond to α-H and β-H of the cysteinate, respectively. In order to confirm these results, we repeated the same experiment on cyt. P450lin which is another bacterial cyt. P450 catalyzing the 8-CH_3

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PII S0014-5793(97)00995-2

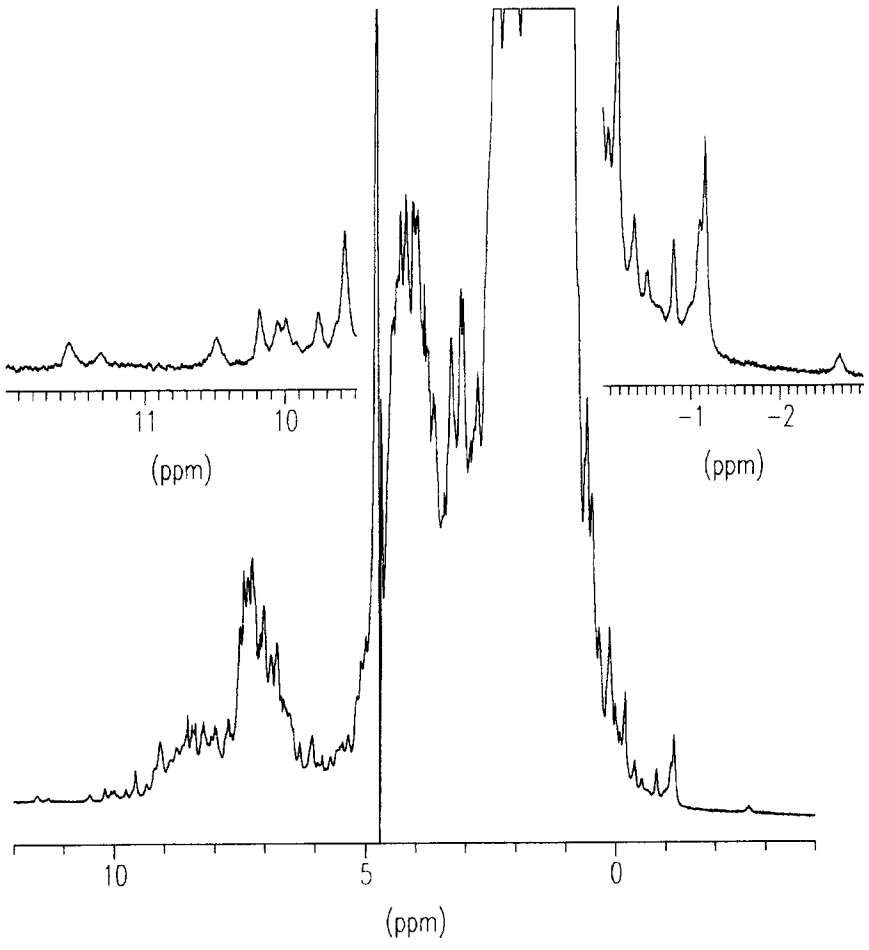

Fig. 1. Full ^{1}H-NMR spectrum with expansions of the low- and high-field regions of reduced cyt. P450cam-CO at 313 K in 100 mM potassium phosphate D_2O buffer, pD 7.

position of linalool [19]. Fortunately, after saturation of the signal at −2.77 ppm with the same pulse duration (50 ms), two well-separated resonances at −0.41 and −0.57 ppm are observed (Fig. 3b). The specific assignment of the two former signals is confirmed by NOE build-up analysis which shows a different behaviour for these two resonances (data not shown): the cross-relaxation rate of the geminate proton (the cysteinate β-H) is much larger than that of the vicinal proton (the cysteinate α-H). Although no crystal structure informations are known about cyt. P450lin, we assume that the cysteinate protons are located in the same position inside the heme pocket of the two proteins. Thus, in the cyt. P450cam spectrum, the resonance at −2.67 ppm arises from cysteinate β′-H. Signals detected at −0.62 and −0.52 ppm originate, respectively, from the cysteinate geminate β-H and vicinal α-H. These assignments are in good agreement with

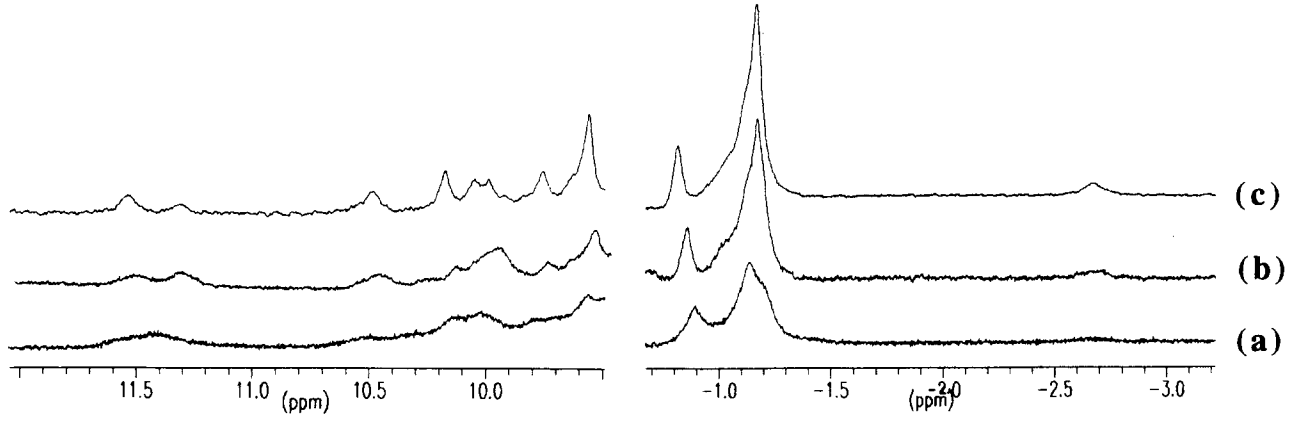

Fig. 2. ^{1}H-NMR expanded spectra (low- and high-field portions) of reduced cyt. P450cam-CO as a function of temperature in 100 mM potassium phosphate D_2O buffer, pD 7. (a) 288 K, (b) 303 K, (c) 313 K.

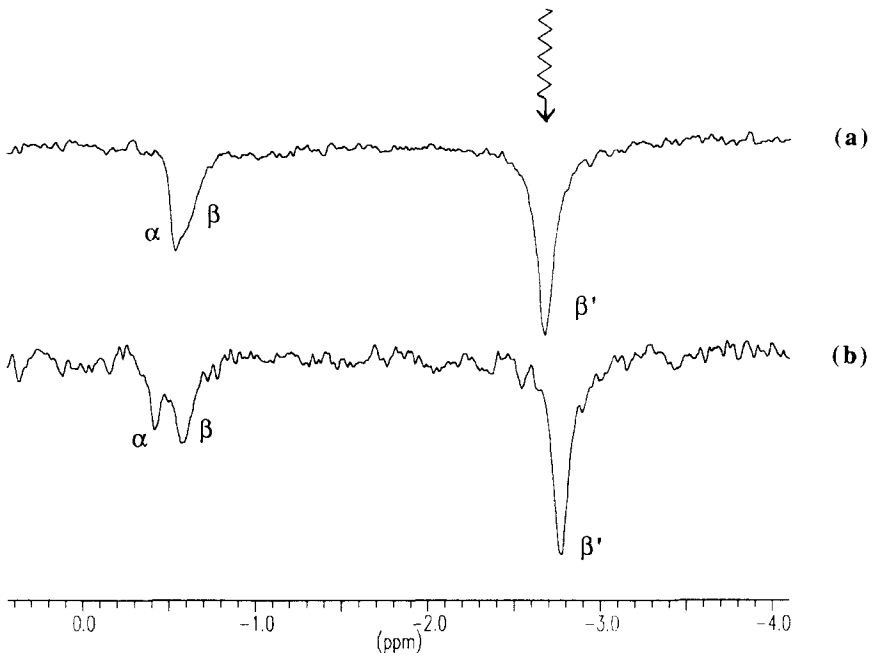

Fig. 3. NOE-difference spectrum of (a) reduced cyt. P450cam-CO and (b) reduced cyt. P450lin-CO at 313 K in 100 mM potassium phosphate D_2O buffer, pD 7. Pre-irradiation of the signals at −2.67 ppm (a) and −2.77 ppm (b) with a pulse of 50 ms duration.

previous results obtained with other sulfide ligands such as methionine in cytochrome *c* [20] or phenethyl mercaptan in one ferrous-porphyrin model [21].

Assignment of the heme protons is realized thanks to the connectivities detected in the 2D-NOESY spectrum (Fig. 4). This spectrum contains several sharp cross-peaks between 9.4 and 10.3 ppm that represent various dipolar interactions between the *meso*-protons and protons of the methyl and vinyl substituents of the heme moiety. A more precise analysis of the spectrum results in the detection of three intense signals at 10.18, 9.76 and 9.58 ppm, presumably due to three *meso*-protons.

The column at 10.18 ppm contains strong NOEs onto two heme methyl groups at 3.27 and 3.46 ppm. Based on the chemical structure of the porphyrin, the only *meso*-proton which can interact with two methyl groups (1-CH_3 and 8-

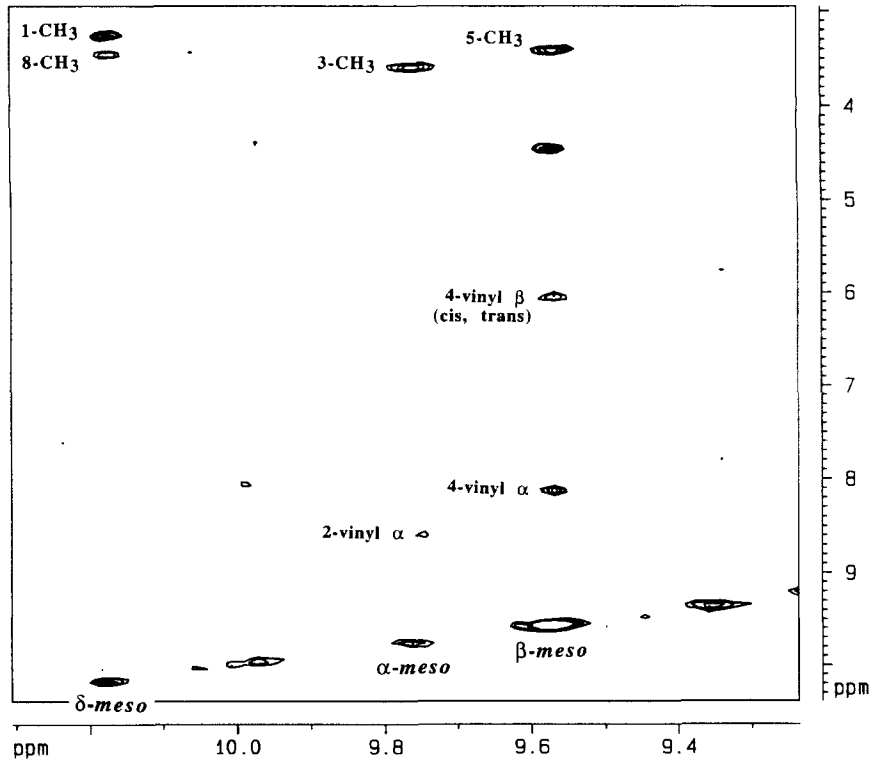

Fig. 4. Down-field region of 500 MHz ^{1}H-NOESY map of reduced cyt. P450cam-CO at 313 K in 100 mM potassium phosphate D_2O buffer, pD 7.

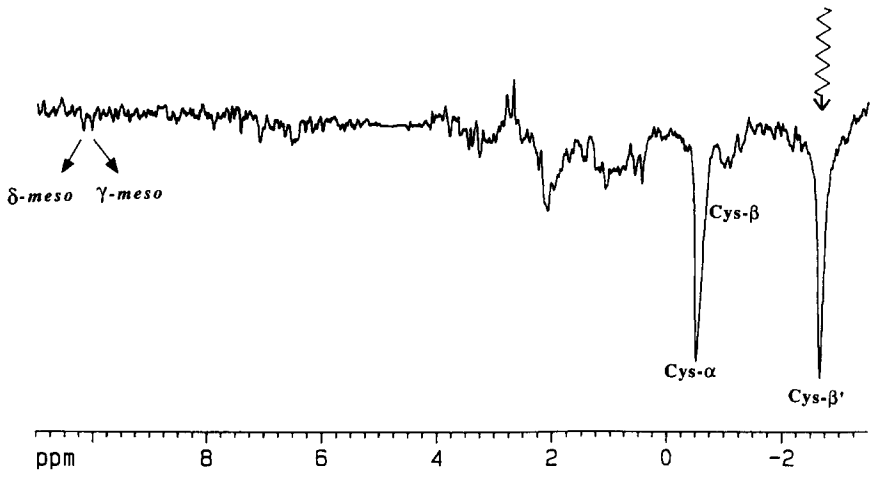

Fig. 5. NOE-difference spectrum of reduced cyt. P450cam-CO at 313 K in 100 mM potassium phosphate D_2O buffer, pD 7. Pre-irradiation of the cysteinate β'-H signal with a pulse of 200 ms duration.

CH_3) is δ-*meso*. Consequently the resonance at 10.18 ppm must arise from the δ-*meso*-proton and the 1-CH_3 and 8-CH_3 resonate at 3.27 and 3.46 ppm but no specific assignment can be made at this step.

At 9.76 and 9.58 ppm two other *meso*-protons are detected, in dipolar interactions with one α-vinyl and one methyl substituents. Analysis of the column at 9.58 ppm indicates strong NOEs with one heme methyl resonance at 3.42 ppm and with one vinyl α-CH resonance at 8.13 ppm. Similar connectivities are noted for the column at 9.76 ppm which displays cross-peaks at 3.60 and 8.58 ppm. The resonances at 9.58 and 9.76 ppm must arise from either the α- or β-*meso*-proton. In the case of the *meso*-proton at 9.58 ppm, two additional weak NOEs are observed at 6.05 and 6.08 ppm. The assignment of these resonances to vinyl β-protons is confirmed by the 2D-COSY spectrum (not shown) which shows connectivities between the vinyl α-proton at 8.13 ppm and these two vinyl β-CH. Moreover, each of these two vinyl β-protons displays in the 2D-NOESY spectrum a cross-peak with a methyl group at 3.60 ppm. This methyl substituent is different from the 1-CH_3 group located at 3.27 or 3.46 ppm (vide supra). The only other methyl group closed to vinyl β-protons is the 3-CH_3, so we assign the resonance at 3.60 ppm to 3-CH_3. Consequently, the signal at 8.13 ppm can be assigned to the 4-vinyl α-proton and the *meso* at 9.58 ppm to the β-*meso*-proton. The remaining vinyl α-proton resonance at 8.58 ppm corresponds to the 2-vinyl α-proton and the α-*meso*-proton resonates at 9.76 ppm. Then the resonance at 3.42 ppm must arise from the 5-CH_3 substituent in dipolar interaction with the β-*meso*-proton at 9.58 ppm.

Finaly, the 2-vinyl α-proton is also in scalar correlation with two vinyl β-CH at 5.66 and 5.47 ppm (COSY spectrum not shown). These protons give a dipolar connectivity with a methyl at 3.27 ppm which shows a cross-peak with the δ-*meso*-proton. Therefore, the resonance at 3.27 ppm is assigned to 1-CH_3 and the remaining 8-CH_3 group is located at 3.46 ppm.

Assignment of the γ-*meso*-proton is proposed thanks to the NOE-difference spectrum obtained upon pre-irradiation of the cysteinate β'-H at −2.67 ppm with a pulse of 200 ms duration (Fig. 5). This spectrum shows weak NOEs at 10.18 and 10.06 ppm. The first resonance at 10.18 ppm arises from the δ-*meso*-proton, as indicated above. Thus, the signal detected at 10.06 ppm originates from the γ-*meso*-proton. This assignment is proposed through the structure of the heme pocket represented in Fig. 6. Indeed, considering the proximity of the cysteinate protons relative to the γ- and δ-*meso*-protons, it is not surprising to observe NOEs between the axial ligand and

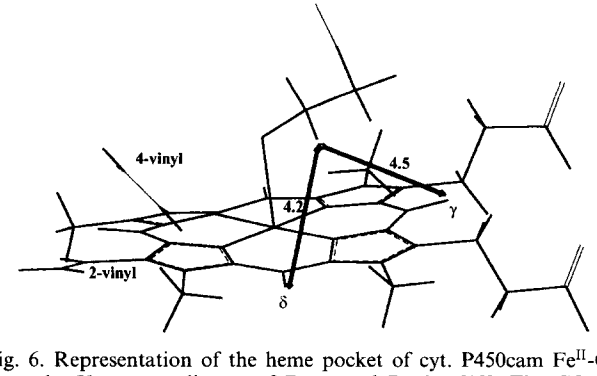

Fig. 6. Representation of the heme pocket of cyt. P450cam Fe^{II}-CO from the X-ray coordinates of Raag and Poulos [18]. The CO ligand was omitted for clarity.

Table 1
Assignments for the diamagnetic cyt. P450cam and P450lin

Assignment	δ (ppm)	
	P450cam	P450lin
Cysteinate protons		
α-H	−0.52	−0.41
β-H	−0.62	−0.57
β'-H	−2.67	−2.77
Heme protons		
α-*meso*	9.76	
β-*meso*	9.58	
γ-*meso*	10.06	
δ-*meso*	10.18	
1-CH_3	3.27	
3-CH_3	3.60	
5-CH_3	3.42	
8-CH_3	3.46	
2-vinyl α	8.58	
β cis, β trans	5.47, 5.66*	
4-vinyl α	8.13	
β cis, β trans	6.05, 6.08*	

*No specific assignment has been performed.

Table 2
^{1}H-NMR chemical shifts for the cysteinate β′-H of cyt. P450cam-CO as a function of various substrates

Substrate	δ ^{1}H (ppm)
No substrate	−2.88
TMCH[a]	−2.71
D-Camphor	−2.67
L-Camphor	−2.61
Fenchone	−2.71
Norcamphor	−2.80
Camphane[b]	−2.59
	−2.86

[a]TMCH: tetramethylcyclohexanone.
[b]Two conformations have been detected for camphane; see Section 4.

these *meso*-protons. Assignment of the heme and cysteinate protons is summarized in Table 1.

Because the cysteinate β′-H is well shielded at −2.67 ppm outside the crowded 0–10 ppm region, we investigated the proton spectra for the diamagnetic cyt. P450cam Fe CO in the presence of various substrate analogues. We have recently shown the influence of the substrate on the ^{13}C-NMR chemical shift for the ^{13}CO-ligand [14]. Table 2 collects the proton chemical shift data of the cysteinate β′-H as a function of the substrate bound to cyt. P450cam. Small chemical shift differences are observed between the cysteinate proton resonances of cyt. P450cam and those of cyt. P450lin (Fig. 3a,b). However, the chemical shifts differ according to the nature of the substrate, with values varying between −2.59 and −2.86 ppm. Moreover, we observe two signals at −2.59 and −2.86 ppm in the presence of camphane, due to different conformers of the substrate in the heme pocket.

4. Discussion

^{1}H-NMR spectroscopy is a powerful tool for the structural study of proteins, but in the case of large proteins, such as cyt. P450, the full analysis of the NMR spectrum is precluded. Nevertheless, in hemoproteins, many resonances can be spread out of the crowded region between 0 and 10 ppm. The shifts originate from the unpaired electron(s) of iron in the paramagnetic forms [1–4] and/or from the ring current of the porphyrin in the diamagnetic forms [21–25]. It can be emphasized that in both cases the protons involved are located inside the heme pocket, close to the metal or the macrocycle. Thus, they correspond to the most interesting protons for structural analysis of the active site.

At 277 K, cyt. P450cam displays a dramatic ^{1}H-NMR spectrum. The temperature increase leads to a nice proton spectrum in regard to line widths. Such an effect is also observed with a lesser extent with cyt. P450lin, suggesting that cyt. P450cam displays larger intermolecular interactions at the NMR range concentration. Moreover, the large increase of the deuterium exchange rate at 313 K permits to simplify spectra in regard to the amide protons as well as for most of the exchangeable protons. The proton NMR spectra of cyt. P450cam Fe^{II}-CO saturated with camphor, under CO atmosphere, in phosphate buffer and highly concentrated, show no change with time. A slight precipitation appears but only a few percent of inactivation occurs as judged by UV-visible spectra.

Obviously, the closest amino acid to the porphyrin ring is the cysteinate fifth ligand, and we can expect a large upfield shift of the corresponding proton signals. Such an expectation was already proposed based on the chemical shifts of model compounds [21]. The broad signal at −2.67 ppm corresponding to the β′-H of the cysteinate ligand is in dipolar connection with its geminate proton at −0.62 ppm and the α-H at −0.52 ppm. Because the transversal relaxation time T_2 is short (≈10 ms), these NOEs are best visualized using 1-dimensional experiment rather than NOESY spectrum. The proposed assignment is confirmed by the mean of the cyt. P450lin study. This last protein fortunately displays two well-separated signals for the β- and α-protons. The chemical shifts observed for diastereotopic β- and α-protons are very similar to the ones of methionine in cytochrome *c*, if we consider the protons in the same position relatively to the sulfur atom [20].

With the exception of the propionic side chains, heme protons have been fully assigned using 2-dimensional NMR spectroscopy. This assignment is based mainly through the NOE cross-peaks of the *meso*- and β-vinyl protons. In the case of one *meso*-proton, the dipolar connectivities concern two groups belonging to different pyrroles whereas for the β-vinyl protons the dipolar interactions are observed with the methyl of the adjacent pyrrole. The crystallographic structure of cyt. P450cam Fe^{II}-CO [18] was helpful for assignment of the γ-*meso*-proton. It is based on the proximity in the crystal structure of the cysteinate β′-proton relative to the γ-*meso*- and δ-*meso*-protons at 4.2 and 4.5 Å, respectively. Evidence for analogy between solution and solid-state structures is obtained by analysis of heme vinyl group orientations. At first, the two α-vinyl protons give large NOEs with the adjacent *meso*-proton accordingly with a rotated or in-plane *cis*-conformation. The absence of NOE cross-peaks, between the α-*meso*-proton and two 2-vinyl β-CH is consistent with a vinyl group almost coplanar with the porphyrin ring (Fig. 6). For the 4-vinyl group the pattern of the connectivities is largely different with the presence of NOEs between the β-*meso*-proton and the two 4-vinyl β-CH. Such NOEs required the vinyl group to be rotated out of the plane of the heme, allowing shorter distances between the *meso*-proton and the vinyl β-CH (Fig. 6). Such a good agreement between the X-ray structure and solution state has already been observed in the case of leghemoglobin and sperm whale myoglobin [22].

The nature of the fifth ligand of cyt. P450s has been discussed for a long time, but the cysteinate is now well established. The large ring current shift induced by the porphyrin ring, which is able to strongly shield a β′-proton at −2.67 ppm, permits us to get a good probe for the coordination sphere of the iron. In this report, we use this NMR probe to show, if any, the influence of the substrate bound in the distal side. Whereas the structural variations induced by various substrates are generally believed to concern mainly the distal side, the chemical shift of the cysteinate β′-proton varies from −2.59 to −2.86 ppm. Obviously, in regard to the distance or orientation of this proton relative to the porphyrin plane, no large structural rearrangments are necessary to explain the observed variations of the chemical shift due to the porphyrin current shift. A slight rotation of the heme plane relative to the fifth ligand or some structural modifications through the polypeptide chain may be involved to explain these chemical shift variations. The high sensitivity of this signal chemical shift also permits to detect the presence of

two conformations for the bound camphane with a splitting of the signal at −2.59 and −2.86 ppm. A splitting of the ^{13}CO resonance for cyt. P450cam Fe^{II}-CO in the presence of camphane has already been observed [14]. Furthermore, crystal structure studies indicate a high mobility of the substrate, presumably due to a non-complete occupancy of the camphane inside the active site [26]. The existence of two hydroxylation sites in the case of camphane which had been observed by Atkins and Sligar [27] agrees well with two possible conformations.

In conclusion, we present herein the first heme proton assignment of the diamagnetic cyt. P450cam. No large variations of the heme proton chemical shifts are observed in comparison with other smaller hemoproteins, also studied in the diamagnetic form. A good agreement between solid-state and solution structures about the vinyl group orientation is demonstrated. The non-exchangeable protons of the fifth cysteinate ligand are shown to be sensitive to the distal modifications induced by the binding of various substrates.

Acknowledgements: We would like to thank Dr. J. de Certaines for 500 MHz NMR facilities, Dr. E. Gill for the preparation of substrates, Dr. K.L. Schröder for running the fermentor culture of *Escherichia coli*, A. Monpert for computer assistance and Prof. I.C. Gunsalus for providing cytochrome P450lin. The work was supported by the European Commission (BIO2-CT94-2060).

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