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Substrate interactions in cytochrome P-450: correlation between carbon-13 nuclear magnetic resonance chemical shifts and C–O vibrational frequencies

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Abstract **^{13}CO NMR chemical shifts and ^{12}CO infrared stretching frequencies have been measured for cytochrome P-450cam–CO in the presence of D-camphor and of various camphor analogues. A linear correlation between both parameters $\delta(^{13}C)$ and $\nu(CO)$ was found indicating that the steric and electrostatic interactions acting on the CO ligand are influenced by the substrate. It has been proved that P-450 complexes are on another line in this correlation than hemoglobins which is explained by the different proximal ligand.**

Key words: Cytochrome P-450; Substrate interaction; ^{13}C NMR; FT-IR

1. Introduction

Measurements of the nuclear magnetic resonance chemical shifts of ^{13}CO hemoprotein adduct have been a powerful method of probing heme electronic structure. However, most of the NMR work has been applied to hemoglobins and myoglobins of various species [1–5]. All these ^{13}C NMR spectroscopic results implicate histidine as an axial ligand. There have, to date, been only two ^{13}CO NMR studies of hemoproteins with cysteinyl mercaptide axial ligand: a ^{13}C NMR study of carbon monoxide complex of ferrous chloroperoxidase, reported by Goff et al. [6], together with unpublished results on bacterial cytochrome P-450cam from Matwiyoff and Philson, reported by Berzinis and Traylor [7]. We present here ^{13}C NMR studies of the carbon monoxide ligand in reduced cytochrome P-450cam (CP101), in the absence or presence of D-camphor, and in the presence of five different camphor analogues in order to evaluate the substrate influence toward the ^{13}CO chemical shifts. A good correlation between $\delta(^{13}C)$ and $\nu(CO)$ is also reported even if the substrates span a wide range in terms of different steric properties.

2. Materials and methods

Cytochrome P-450cam (CYP101) was expressed in *Escherichia coli* and purified to an absorbance ratio 392 nm/280 nm of 1.22 as previously described [8]. Camphor was removed by dialysis against 50 mM Tris-HCl, pH 7.4, with a final run of a Sephadex G25 (medium) column equilibrated with the same buffer. The eluted substrate-free P-450 was dialyzed extensively against 100 mM D_2O phosphate buffer (pH 7) and concentrated by ultrafiltration to a final protein concentration of about 1.2 mM.

Camphor (D and L forms), norcamphor, 3,3,5,5-tetramethylcyclohexanone (TMCH) and fenchone were obtained from Aldrich. Camphane was prepared by the Wolff-Kichner reduction of camphor semicarbazone [9]. Each substrate was dissolved in deuterated methanol. Addition of 10 μl of this stock solution to 500 μl of cyt P-450 solution lead to a final substrate concentration of 2 mM which was sufficiently high to guarantee a complete substrate binding. ^{13}CO (99%) was purchased from Aldrich. Samples were flushed first with argon and then with labelled carbon monoxide. After reduction of the protein by injection of a small aliquot of sodium dithionite D_2O solution, corresponding to a tenfold excess of dithionite to P-450, the protein solutions were transferred to the NMR tubes previously flushed with ^{13}CO. Each P-450–CO complex was checked in the Soret band region using the UVIKON 941 spectrophotometer.

Carbon-13 NMR spectra were recorded on a Bruker DMX 500 spectrometer at 125.7 MHz frequency with a 5 mm dual ^{13}C,^{1}H probe. A sweep width of 32.7 kHz was employed along with 60° flip angle pulse and 0.55 s repetition time. Proton decoupling was applied during all the experiments. Around 100,000 scans were acquired with a total acquisition time of 15 h per spectrum. The chemical shifts were referenced through an external capillary containing tetramethylsilane. The cyt P-450 sample was not changed during this long acquisition time as seen from the identity of the optical spectrum and the proton NMR spectrum before and after the ^{13}C NMR measurement.

Infrared spectra were run on the Fourier-transform infrared spectrometer IFS66 (Bruker) with a liquid nitrogen-cooled mid-band MCT detector at 2 cm^{-1} resolution. The samples were prepared from the same protein solution (D_2O buffer) used for the NMR study, however, ^{12}CO gas was taken for the CO complex. The single channel spectrum of the P-450–CO sampled was ratioed against the oxidized P-450 as background. Spectra were taken in the double-sided/forward–backward acquisition mode. Fourier transformation of the interferogram was performed with a zero-filling factor of 2 and the Blackman-Harries 4-point apodization function. 200 interferograms were accumulated. The protein sample was placed between two CaF_2 windows separated with a Teflon spacer of 100 μm thickness. All infrared spectra were corrected from the water vapor. Baseline correction and curve fitting with the Voigt function were performed using a curve fit software package as previously described [8,10].

3. Results

The ^{13}C NMR spectrum of carbonyl cyt P-450cam, in the presence of L-camphor, in D_2O buffer, is illustrated in Fig. 1a. The broad singlet at 200.7 ppm is due to the coordinated ^{13}CO. Replacement of L-camphor by D-camphor yields spectrum b in Fig. 1. The signal at 200.4 ppm is also assigned to CO bound to heme Fe(II). These values are in agreement with the region of chemical shifts previously reported for camphor-bound cyt P-450cam [7] and chloroperoxydase [6]. Similar results have been obtained in our studies with other substrate analogues (Fig. 1). Table 1 collects the chemical shift data for CO-bound

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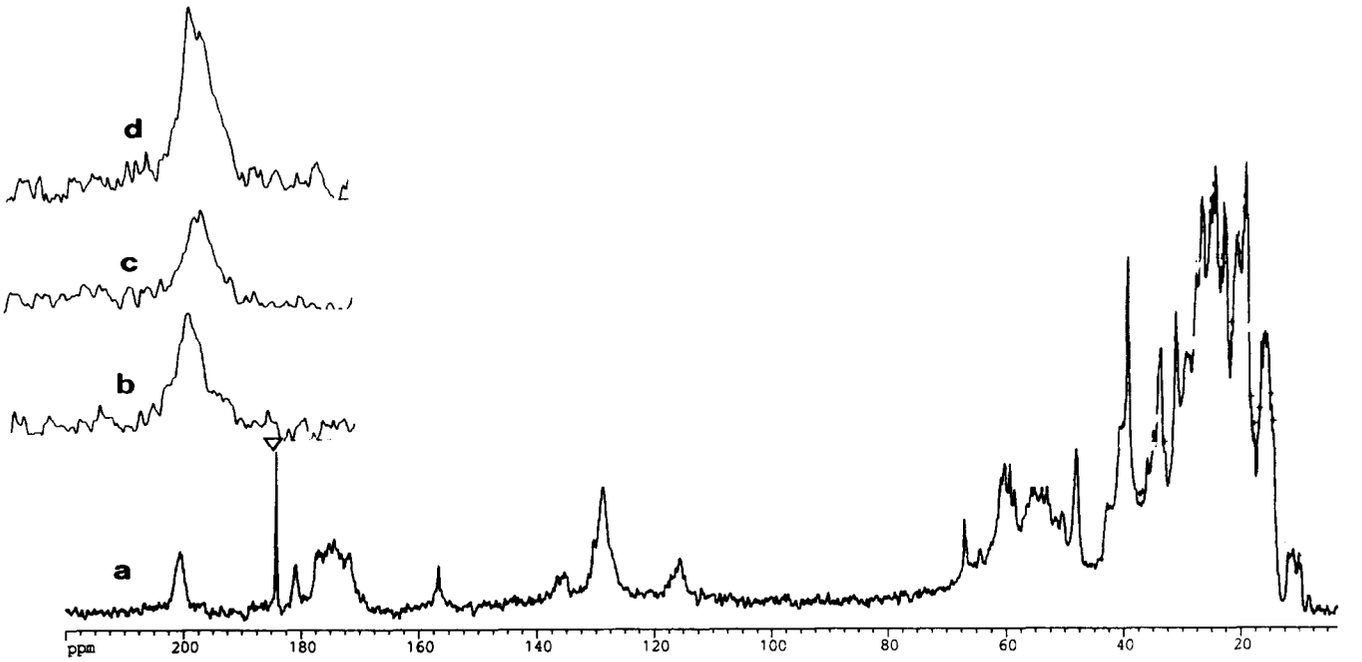

Fig. 1. Proton-decoupled carbon-13 NMR spectra of ferrous cyt P-450cam–CO (a) in the presence of L-camphor (complete spectrum), (b) in the presence of D-camphor (low-field portion of the spectra), (c) substrate-free and (d) in the presence of camphane. The asterisk denotes free ^{13}C inside the solution.

cyt P-450 in the presence of various substrate analogues. All of them show a broad resonance at about 200 ppm downfield from tetramethylsilane. The corresponding CO stretching modes are also summarized in Table 1 and Fig. 2. The frequencies are very similar to the previously published values found for the cyt P-450 samples in 100 mM potassium phosphate in H_2O, pH 7, 20% glycerol [8]. Because of the absence of glycerol all samples exhibit a small cytochrome P-420 contribution of approximately 10% estimated from the Soret band which causes the infrared band at about 1963 cm^{-1}. This P-420 contribution is too small to be detectable as a separate resonance in the ^{13}C NMR spectra. In the substrate-free protein this cyt P-420 peak overlaps with the 1962–1964 cm^{-1} band for the native protein and corresponds to 10% of this band [8]. The substrate-free protein also shows a changed population distribution between the different conformers [8] due to the missing glycerol.

Although the ^{13}C chemical shifts for the different substrate analogue P-450 complexes appear in a narrow range around 200 ppm there is a clear linear relation between the distinct $\delta(^{13}C)$ values and the ^{12}CO stretching mode frequencies (Fig. 3A). Such correlation between ^{13}CO chemical shift and the ^{12}CO stretching frequency has been shown recently for hemoglobins and myoglobins [11–13]. However, this type of hemoprotein follows another line (eq. 1, Fig. 3A).

$$\delta(^{13}C) = -0.045\ \nu(CO) + 287.2 \quad \text{(corr. coeff. = 0.600) cyt P-450}$$
$$\delta(^{13}C) = -0.0705\ \nu(CO) + 344.3 \quad \text{(corr. coeff. = 0.754) hemoglobins [11]} \qquad (1)$$

A similar linear correlation is found with our ^{13}C NMR data and the iron–CO stretch frequencies ν(Fe–C) obtained by

Table 1
^{13}C NMR chemical shifts, ^{12}CO and Fe–C stretch frequencies for cyt P-450cam–CO and chloroperoxidase

Substrate	$\delta(^{13}C)$	$\nu(CO)$	$\Delta\nu_{1/2}$	Population	ν(Fe–C)
1. TMCH	200.86	1933.4	8.9	0.80	485 [14]
		1939.9	18.4	0.20	
2. D-Camphor	200.40	1940.2	12.2	1.00	481 [15]
3. L-Camphor	200.70	1941.4	10.2	1.00	
4. Fenchone	200.08	1944.1	10.8	1.00	480 [14]
5. Norcamphor	199.83	1946.4	9.8	1.00	473 [15]
6. Camphane	200.03	1942.9	19.4	0.29	
	199.40	1951.9	10.8	0.71	
7. Free	199.91	1938.4	18.6	0.44	
		1953.1	11.1	0.05	
	199.56	1962.5	14.3	0.51	464 [15]
8. Camphorquinone	–	1941.0 [8]	10.7	0.86	476 [14]
9. Adamantone	–	1942.3 [8]	9.1	1.00	474 [15]
10. Chloroperoxidase pH 6	200.8 [6]	1958.0 [32]	15	–	484 [31]

$\delta(^{13}C)$ in ppm; ν, $\Delta\nu_{1/2}$ in cm^{-1}; population, conformer population.

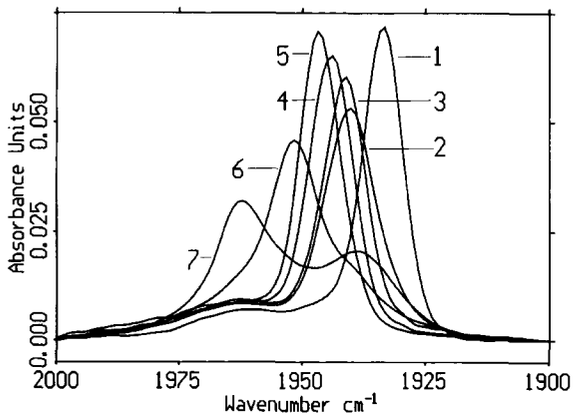

Fig. 2. CO stretch infrared spectra of cyt P-450cam–CO in the presence of camphor and its analogues and for substrate-free P-450. 1, 3,3,5,5 tetramethylcyclohexane; 2, D-camphor; 3, L-camphor; 4, fenchone; 5, norcamphor; 6, camphane; 7, substrate free.

others [14] from resonance Raman measurements (Fig. 3B). The corresponding data for the hemoglobins were taken from the literature [12]. Regression analysis gives:

$\delta(^{13}C) = 0.056\ \nu(Fe–C) + 173.2$
(corr. coeff. = 0.851) cyt P-450
$\delta(^{13}C) = 0.093\ \nu(Fe–C) + 159.7$
(corr. coeff. = 0.587) hemoglobins [12] **(2)**

4. Discussion

Hemoproteins span a broad range of functions in organisms; all of them require the interaction of small ligands, like for example dioxygen, with the heme iron. Therefore, there is considerable interest in the nature of the Fe–ligand bonding in hemoproteins, in particular of the Fe–CO bonding as a model. For cytochrome P-450 it has been shown by the crystal structure analysis [16] that the substrate binds near the heme group. A significant influence of the substrate and substrate analogues has been observed on the ν(CO) stretching mode which allowed the proposition of different Fe–CO geometries and/or alterated polarity effects [8,10]. At least for myoglobin an alterated Fe–CO geometry for the subconformers with the ν(CO) A_0–A_3 has been put in question recently, based on new crystal structure data and infrared circular dichroism studies [17]. The electrostatic field near the Fe–CO group is discussed as a relevant parameter which determines the polarization of the CO ligand and the shielding/deshielding of the ^{13}C nucleus reflected in the CO stretch frequency as well as in ^{13}C NMR chemical shifts [12,19].

According to Ramsey [20], Karplus and Das [21] and Pople [22] the total chemical shielding σ of a nucleus A is given by three terms: (i) the first order contribution to the shielding tensor, the so-called diamagnetic shielding σ^d_A, which depends on the electron density on A and is always a positive value; (ii) the second order contribution, the so-called paramagnetic shielding σ^p_A, which mainly represents the effect of bond parameters and is expected to be a negative term; and (iii) σ' which includes all other effects like ring currents.

$\sigma = \sigma^d_A + \sigma^p_A + \sigma'$

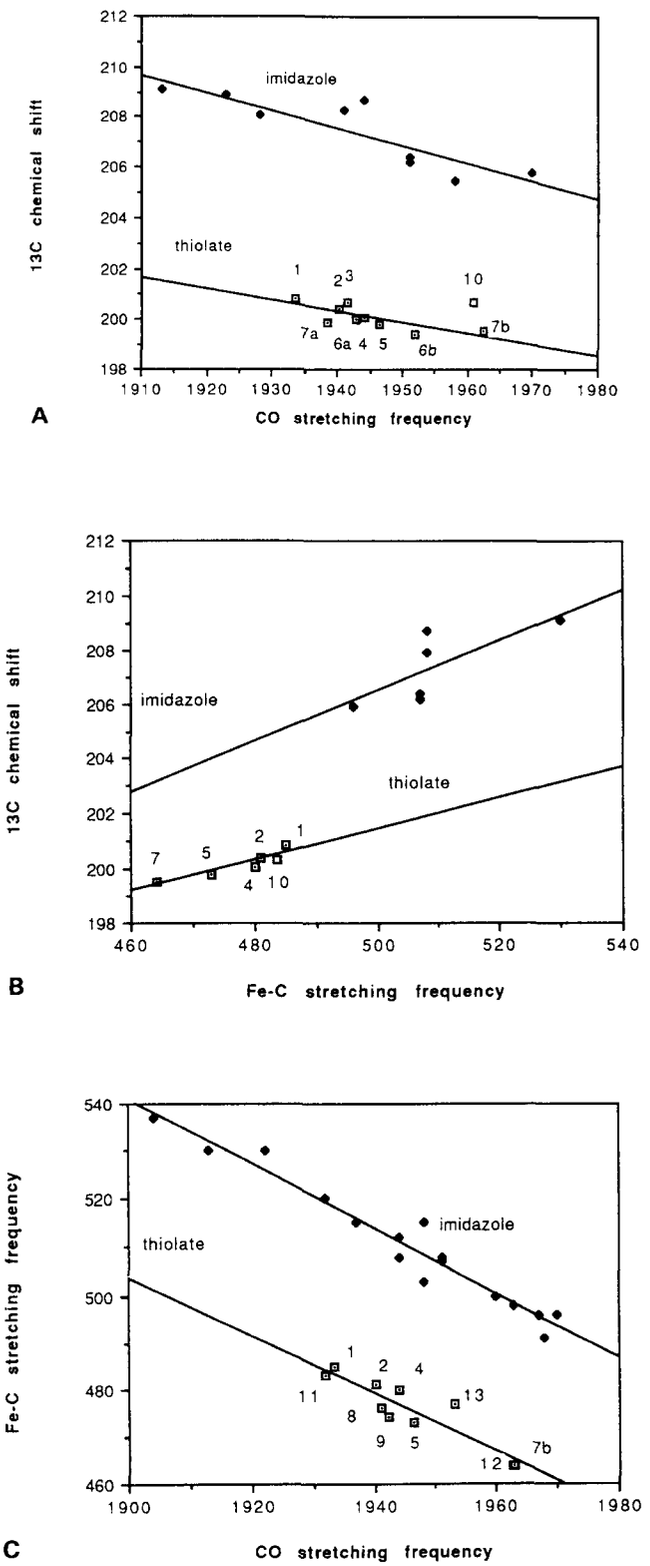

Fig. 3. Correlation of ^{13}CO NMR chemical shifts and stretch frequencies for cyt P-450cam and chloroperoxidase compared to hemoglobins. (A) ν(CO); (B) ν(Fe–C); (C) ν(CO)/ν(Fe–C). 1, 3,3,5,5 tetramethylcyclohexane; 2, D-camphor; 3, L-camphor; 4, fenchone; 5, norcamphor; 6a,b, camphane conformers; 7a,b, substrate free; 8, camphorquinone [8,14]; 9, adamantanone [8,15]; 10, chloroperoxidase pH 6 [6,31,32]; 11, cyt P-450 putidaredoxin [25]; 12, cyt P-450 bovine adrenocortical mitochondria [25]; 13, cyt P-450cam 464 [25]. The values for the hemoglobins were taken from [12] and [25].

It has been shown for carbonyl metal complexes [23] that the differences in the ^{13}C NMR chemical shifts in these complexes are mainly contributed by bond parameters that derive from the paramagnetic shielding term.

σ_A^p is given by [24]:

$$\sigma_A^p = [e^2h^2/2m^2c^2\Delta E] < r^{-3} \geq [Q_{AA} + \Sigma_{B \neq A} Q_{AB}] \quad (3)$$

where ΔE is an average excitation energy, Q_{AA} and Q_{AB} are the elements of the charge density/bond order matrix, r represents the mean radius of the 2p orbital at the CO carbon atom.

According to Buchner and Schenk [23] the term in brackets in eq. 3 can be substituted by:

$$Q_C + Q_{CO} + Q_{Fe\text{-}C} = 4/3[1 - P^\sigma(C\text{–}O)P^\pi(C\text{–}O) - 3^{1/2}p^\sigma(Fe\text{–}C)P^\pi(Fe\text{–}C)] \quad (4)$$

P^σ and P^π are the bond orders for the σ and the π part, respectively, for the C–O or Fe–C bond. Resonance Raman studies have shown that there is an inverse linear correlation between ν(CO) and ν(Fe–C) [25]. In Fig. 3C, we have plotted literature data of ν(CO) and ν(Fe–C) for hemoglobins [25] and of ν(Fe–C) [14,15] and our ν(CO) for cyt P-450cam. This linear correlation has been explained by the ligand-σ-donation and iron-π-backdonation phenomenon, the most important parameter being the π-bond order [26]. Because the bond order products for the C–O and the Fe–C bond contribute to eq. 4 with the same sign, one can expect that ν(CO) and ν(Fe–C) should correlate with the ^{13}C chemical shift in an inverse manner, which is also the case (Fig. 3A and B).

Two important structural pieces of information can be obtained from these correlations. (i) Complexes with different proximal ligands form different lines. Park et al. [12] propose that for the correlation between $\delta(^{13}C)$ and ν(Fe–C) the complexes with imidazole and cysteinyl mercaptide (thiolate) should be on the same, but non-linear curve. This conclusion was drawn from data of only three thiolate complexes (chloroperoxidase, cyt P-450cam, thiolate heme model complex). However, our studies of the different P-450 substrate complexes clearly show that P-450 and hemoglobins are on different lines. This finding can be explained by the different effect of the imidazole and thiolate proximal ligand on the electron density distribution in the heme complex. Thiolate donates more σ-electron density to the iron than imidazole, as can be concluded from the lower lone-pair orbital ionization potential for S^- (5.49 eV) compared to 15.08 eV for N (Imidazole) [27,28]. Thus, increased σ-donation to Fe from the cysteinate ligand in P-450cam seems responsible for the increased shielding. (ii) The fact that all complexes with thiolate are on one line strongly suggest that no differences in the 5th ligand property exist for the different substrate analogue P-450 complexes. The different ν(CO), ν(Fe–C) and $\delta(^{13}C)$ along each line reflect effects from the distal side, as it has been suggested also by others [12,25].

Obviously, the different substrates bound in the heme pocket of cyt P-450cam influence the electric field near the CO ligand. From the present study we cannot suggest whether this influence is directly induced by the substrate–CO ligand contact or indirectly by the substrate induced conformational change of the protein which leads to different contacts between the CO ligand and amino acid residues or different inclusion of water molecules in the heme pocket.

We should also note that two overlapping ^{13}C NMR signals are observed for camphane complexation to P-450cam which we assign to the two overlapping infrared bands (Fig. 1, Table 1). Camphane does not form an hydrogen bond to tyrosine-96 OH group as the natural substrate camphor does [15,29]. This allows a higher mobility and a disordered binding of the substrate analogue in the heme pocket which may cause the disorder of the CO ligand geometry [8]. Such high mobility has been recently reported, using CO recombination kinetic experiments of cyt P-450 in the presence of various substrates [30]. For substrate-free cyt P-450 two ^{13}C NMR resonances are also observed which we assign to the two major CO stretch bands in the infrared spectrum (Fig. 2, Table 1). Water molecules in the heme pocket have been suggested to induce the different conformers [8].

In conclusion, our results presented here demonstrate that the measurements of ^{13}CO NMR chemical shifts of various substrate-bound cytochrome P-450–CO are very sensitive to the nature of the proximal ligand and also sensitive to the nature of the substrate. Thus, the ^{13}C chemical shift may provide a new probe for substrate bonding to cytochrome P-450cam. Such results should also give a standard with which to compare future results from other members of the cytochrome P-450 family.

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