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Direct observation of substrate binding to ferrous-CO cytochrome P-450-CAM using ^{19}F NMR

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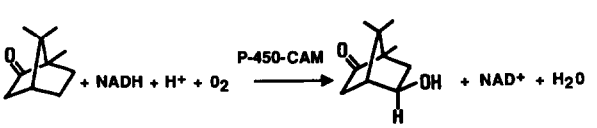
The binding of two fluorinated substrate analogs, 9-fluorocamphor and 5,5-difluorocamphor, to cytochrome P-450-CAM has been investigated by ^{19}F NMR spectroscopy. The NMR properties of each substrate differ depending on whether it is free in aqueous buffer, bound to the diamagnetic ferrous-CO enzyme or bound to the paramagnetic ferrous derivative. As CO must be bound to the ferrous center for it to be diamagnetic, these results demonstrate that camphor and CO bind to the protein simultaneously. The present results are unusual in that the spectral properties of the substrate rather than those of the heme iron have been monitored to follow substrate and ligand binding.

NMR, ^{19}F-; Cytochrome P-450; Oxygen activation; Monooxygenase; Substrate binding; Ligand binding

1. INTRODUCTION

The P-450 cytochromes are a family of heme iron enzymes with similar electronic absorption properties that activate dioxygen for incorporation into unactivated hydrocarbons [1-8]. Addition of CO, a potent inhibitor, to the paramagnetic deoxy-ferrous enzyme produces the diamagnetic ferrous-CO state. This species has a Soret electronic absorption band near 450 nm from which the name P-450 was derived. The camphor-metabolizing cytochrome P-450-CAM from *Pseudomonas putida*, the first P-450 to be purified [2], is soluble and has been the frequent choice for physical studies of the P-450 system. P-450-CAM stereo- and regiospecifically converts (1*R*)-(+)-camphor to 5-exohydroxycamphor (scheme 1) [2,4-6]. When the primary site of hydroxylation is completely blocked in 5,5-difluorocamphor, 9-hydroxy-camphor is the product [9]. The stereospecificity of P-450-CAM, often absent from mammalian P-450 [1], coupled with the ability of the enzyme to hydroxylate unactivated substrates increase the importance of understanding the nature of the active site. In this regard, Poulos and co-workers [10-13] have recently reported X-ray crystal structures of ferric P-450-CAM with and without camphor bound.



Scheme 1

The interaction of camphor with ferrous and with ferrous-CO P-450-CAM has been investigated previously. The K_d value for camphor is identical for the two states, suggesting that CO binding does not interfere with substrate binding [14]. The affinity of CO for the ferrous enzyme is, however, diminished 10-fold when camphor is bound [15]. Furthermore, although the Soret electronic absorption band of the ferrous-CO enzyme shifts by only 1 nm upon camphor binding [16], significant

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changes in the energies of the Fe-C and C-O stretches of the ferrous-CO enzyme are observed [17,18]. The perturbations in spectroscopic properties of the Fe-CO unit caused by camphor binding have led to the conclusion that the binding pocket of the enzyme can accommodate camphor and CO simultaneously. The present study addresses this issue from the perspective of the substrate. In particular, we have observed significant differences with the ^{19}F NMR spectrum of two enzyme-bound fluorocamphor substrate analogs (fig.1) in the presence and absence of CO. As in the earlier work, we conclude that camphor and CO bind to the ferrous enzyme concurrently.

2. MATERIALS AND METHODS

All commercially available chemicals were of reagent grade from Aldrich or Fisher. The syntheses of 9-fluorocamphor and 5,5-difluorocamphor have been reported [9,19]. Cytochrome P-450-CAM was isolated from *P. putida* grown on (1*R*)-(+)-camphor as in [20–22]. The concentration of the ferric enzyme was determined from the intensity of the Soret band at 391 nm [20]. Enzyme purity was established from the 391/280 nm absorbance ratio [20–22]; samples with ratios above 1.4 have been found to be electrophoretically homogeneous and were used in this study. Substrate binding was carried out as described [9,22].

A Bruker WH-400 NMR spectrometer with a ^{19}F frequency of 376.13 MHz and controlled by an Aspect 2000A computer was employed. All data were obtained using a 10 mm $^{19}F/^{1}H$ dual-frequency probe. FIDs were collected using 8K word data tables that were zero-filled to 16K words before transforming. The use of 15 000 Hz sweep width gave digital resolution of ~0.9 Hz/pt.

Protein solutions for NMR experiments were concentrated to 2 mM in 0.1 M potassium phosphate buffer, pH 7.4, with 20% D_2O as an internal lock. Sample temperature was maintained at 4°C during handling. Sample volumes of 2.5 ml were used in 10 mm Wilmad Royal Imperial NMR tubes septum-sealed under nitrogen. Ferrous P-450-CAM was prepared in a Vacuum Atmospheres dry box by reduction with a 5-fold excess of $Na_2S_2O_4$. The ferrous-CO state was generated by passing a stream of CO over the ferrous enzyme. Complete reduction and CO coordination were each verified by electronic absorption spectroscopy on an aliquot diluted 100-fold with anaerobic buffer. Protein integrity was checked by examination of the electronic absorption spectrum of the ferrous-CO derivative; essentially no P-420 (inactive P-450 [1–6]) was present at the start and less than 5% at the completion of any experiment.

Fig.1. Structure of the fluorocamphors used in this investigation.

3. RESULTS AND DISCUSSION

Binding of camphor to ferric P-450-CAM converts the heme iron from low- to high-spin. This change, easily detectable by the 27 nm blue-shift in the Soret absorption band, results from displacement by camphor of the distal water ligand to the heme iron [11,13]. Camphor binds to a site adjacent to the heme iron but does not coordinate to the metal [11,13]. Upon CO binding to the ferrous enzyme, a spin state change also occurs, from high to low, and the Soret band shifts from 408 to 446 nm. As discussed above, camphor binding perturbs the spectral properties of the ferrous-CO center [14–18] suggesting that CO and camphor bind to the active site simultaneously. Here, we assess this issue from the perspective of the substrate in order to determine whether CO binding perturbs the spectroscopic properties of the substrate.

The ^{19}F NMR spectrum of 5,5-difluorocamphor in aqueous buffer (fig.2A) is an AB pattern comprised of four resonances. The signals at −10.3 and −11.5 ppm have been assigned to the *exo* fluorine and those at −21.6 and −22.9 to the *endo* fluorine. Each fluorine produces two signals due to scalar coupling to the other fluorine. These assignments have been derived from the ^{19}F and ^{1}H NMR spectra of 5,5-difluorocamphor in d_1-chloroform. In particular, the ^{1}H NMR resonance of the proton at position 4 shows a $J_{H\text{-}F}$ coupling of 6.8 Hz. Only the 5-*exo* fluorine has the appropriate dihedral angle to produce such coupling. The ^{19}F NMR spectrum of 9-fluorocamphor exhibits an AXX′ pattern and has been assigned [19]. Upon binding to ferrous-CO P-450-CAM, new resonances appear for 5,5-difluorocamphor (fig.2B) and 9-fluorocamphor [19]. The new resonances in fig.2B, summarized along with all other NMR data in table 1, correspond to the fluorine(s) of the enzyme-bound fluorocamphors. The changes in chemical shift of 1–2.5 ppm can be attributed to a microenvironmental effect upon substrate binding to the protein, possibly due to the ring current of the heme.

The NMR properties of the two fluorocamphor substrate analogs are again altered upon binding to the ferrous enzyme. For 9-fluorocamphor, the ^{19}F

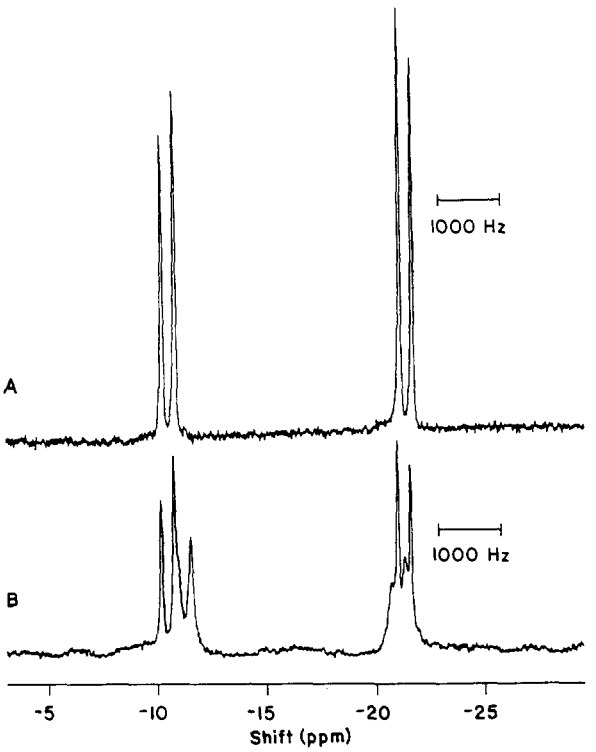

Fig.2. (Top) 376 MHz ^{19}F NMR spectrum of 5,5-difluorocamphor in potassium phosphate buffer, 20% D_2O, pH 6.8. (Bottom) 376 MHz ^{91}F NMR spectrum of excess 5,5-difluorocamphor bound to 2 mM ferrous-CO P-450-CAM in 100 mM potassium phosphate buffer, 20% D_2O, pH 6.8.

resonance shifts another 3.4 ppm from its location in the ferrous-CO case (table 1) and is broadened 100-fold as a result of being in close proximity to the paramagnetic ferrous iron. Quantification of this effect using the Solomon-Bloembergen equation [23,24] to determine an average Fe–F distance of 3.8 Å has been reported [19]. As carbon 5 of camphor is closest to the heme iron of P-450-CAM [11,25], it is perhaps not surprising that no resonances are detected for the fluorines of 5,5-difluorocamphor bound to ferrous P-450-CAM.

The possibility that the fluorocamphors bind to a site other than the active site can be eliminated by examination of the data for 9-fluorocamphor-bound ferrous P-450-CAM. In the presence of excess substrate, two sets of resonances are observed, one from camphor free in solution and a second from camphor bound within the active site (table 1). Binding of substrate to an additional site would have been expected to produce a third set of resonances. The possibility of rapid chemical exchange between bound and free states has been ruled out both by previous studies [26] and by the appearance of separate signals for the bound and free fluorocamphors (table 1). If the fluorocamphors were in fast exchange, a single resonance would appear at a chemical shift corresponding to the mole fraction weighted average of the two chemical shifts. This is clearly not the case as shown in fig.2B.

In summary, the NMR properties of the two fluorocamphor substrate analogs differ depending on whether the probe is free in solution, bound to the paramagnetic enzyme or bound to the diamagnetic state. Since CO must be coordinated for the ferrous iron to be diamagnetic, the substrate and CO must be bound to the enzyme at the same time. The present results complement earlier studies of camphor binding to ferrous-CO P-450-CAM in reaching the same conclusion by

Table 1

Fluorine chemical shifts

Camphor	Species	Chemical shift[a]
5,5-Difluoro-	free in buffer[b]	−10.3[c], −11.5, −21.6, −22.8
5,5-Difluoro-	bound to diamagnetic ferrous-CO P-450-CAM	−11.1[c], −12.3, −20.9, −22.1
9-Fluoro-	free in buffer[b]	−156.6, −156.7, −156.9
9-Fluoro-	bound to diamagnetic ferrous-CO P-450-CAM	−154.1[d]
9-Fluoro-	bound to paramagnetic ferrous P-450-CAM	−150.7[d]

[a] In ppm relative to external trifluoroacetic acid. Data for 9-fluorocamphor have been previously reported [19]

[b] Buffer: 100 mM potassium phosphate, 20% D_2O, pH 7.4

[c] The first pair of signals have been assigned to the *exo* fluorine. Each fluorine gave two resonances due to the $J_{F\text{-}F}$ coupling

[d] The fluorine-proton coupling, $J_{F\text{-}H}$, was smaller than the observed linewidth

monitoring the spectral properties of the substrate rather than those of the ferrous-CO heme iron.

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