^{1}H Nuclear Magnetic Resonance and Magnetic Circular Dichroism Studies of Ferric Low-spin Cytochrome P-450$_{scc}$

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Abstract

400 MHz ^{1}H NMR of ferric low-spin cytochrome P-450$_{scc}$ purified from bovine adrenal cortex was measured for the first time*. As compared with ^{1}H NMR spectra of low-spin P-450$_{cam}$ and metMb–mercaptan complexes, paramagnetic shifts of low-spin P-450$_{scc}$ complexes were more divergent, suggesting that there is a subtle difference in the heme environment between P-450$_{scc}$ and P-450$_{cam}$ [1]. The paramagnetic shifts of low-spin complexes of P-450$_{scc}$ caused by adding nitrogenous inhibitors, aminoglutethimide and metyrapone, were different from those caused by adding an intermediate, 20α-hydroxycholesterol, and a detergent, Tween 20 [2]. The paramagnetic shifts of the metMb–mercaptan complexes were convergent compared with those of ferric low-spin P-450$_{scc}$ and P-450$_{cam}$, suggesting that the electronic character and/or the conformation of the internal thiolate ligand in P-450$_{scc}$ and P-450$_{cam}$ are different from those of the external thiolate ligand in metMb–thiolate complexes [3]. The paramagetic shifts of the metMb–mercaptan complexes were dependent on the electron donating factor of the alkyl group of the bound mercaptans [4].

Magnetic CD(MCD) spectra of ferric low-spin P-450$_{scc}$, rabbit liver P-450 complexes and metMb–mercaptan complexes were also observed at various temperatures*. The temperature dependences of the Soret MCD bands for the low-spin P-450 and metMb-mercaptan complexes were decidedly less pronounced than those for the low-spin metMb–CN$^-$ or imidazole complexes, suggesting that thiolate ligands markedly influence the Soret MCD band of the ferric low-spin complexes [1]. The suggestion described in [2] implied by the ^{1}H NMR study was reconfirmed from the temperature dependence study of the Soret MCD [2]. The temperature dependences of the Soret MCD bands for low-spin P-450 complexes having a non-nitrogenous ligand were more pronounced than for those having a nitrogenous ligand.

Introduction

Cytochrome P-450$_{scc}$ (P-450$_{scc}$) is a thiolate (of cysteine) ligated hemoprotein and contributes to a side-chain cleavage reaction of cholesterol in adrenal cortex mitochondria [1]. P-450$_{scc}$ is peculiar in that addition of non-nitrogenous exogenous effectors such as an intermediate, 20α-hydroxycholesterol, a reaction product, pregnenolone, or a detergent, Tween 20, to the ferric high-spin P-450$_{scc}$ causes low-spin P-450$_{scc}$ complexes [2]. This is in contrast with other P-450 complexes such as P-450$_{cam}$ or rabbit liver P-450, where low-spin ferric complexes are not induced by their intermediates or products [2–5].

We here describe 400 MHz ^{1}H NMR spectra of ferric low-spin P-450$_{scc}$ complexes for the first time and compare these with the ^{1}H NMR spectra for P-450$_{cam}$ [6, 7] and metMb–mercaptan complexes. Since ^{1}H NMR spectra of P-450 purified from animal internal organs have not been reported, the first ^{1}H NMR spectra of P-450$_{scc}$ purified from bovine adrenal cortex should be emphasized here. We also report here a temperature dependence study of MCD spectra of the ferric low-spin P-450$_{scc}$, as well as those of rabbit liver P-450's and low-spin metMb complexes. On the basis of these spectra findings, special electronic characters of ferric low-spin P-450$_{scc}$ or thiolate-ligated heme complexes were deduced.

Experimental

P-450$_{scc}$ was purified homogenously from bovine adrenal cortex mitochondria as described previously

*Abbreviations used are: P-450$_{scc}$, cytochrome P-450 which is purified from bovine adrenal cortex mitochondria and catalizes a side-chain cleavage reaction of cholesterol; Tween 20, sorbitan mono-9-octadecenoate poly(oxy-1,2-ethanediyl) derivative; NMR, nuclear magnetic resonance; MCD, magnetic circular dichroism; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; aminoglutethimide, 3-(4-aminophenyl)-3-ethyl-2,6-piperidinedione; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; P-450$_1$, a major component of low-spin type cytochrome p-450 purified from phenobarbital-induced rabbit liver microsomes; P-448$_1$, a high-spin type cytochrome P-450 purified from phenobarbital-induced rabbit liver microsomes; P-450$_{cam}$, cytochrome P-450 purified from camphor-grown *Pseudomonas putida*; P-420$_{scc}$, a denatured form of P-450$_{scc}$ (*cf.* Experimental).

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[2]. P-450$_1$ and P-448$_1$ were purified homogenously from phenobarbital-induced rabbit liver microsomes as described previously [8–11]. Sperm whale metMb was purchased from Sigma and was used without further purifications. Aminoglutethimide and metyrapone were kindly supplied from Ciba-Geigy. Other reagents used were of the highest guaranteed grade and were used without further purification.

Before each spectral measurement, absorption or MCD spectral titrations were done for all P-450 complexes described in this study to confirm that ferric low-spin complexes were certainly formed by adding effectors [2]. Then NMR spectra were obtained after confirming that the low-spin complexes are formed by adding the effectors for the concentrated protein solutions [0.3–3 mM], with the aid of the absorption spectra measured in a 1 mm cell.

P-420$_{scc}$ was formed by incubation in 60% saturated $(NH_4)_2SO_4$ solution at room temperature for 20 min. The CO–bound ferrous form of this compound did not show a Soret absorption band at 450 nm, but showed only one Soret absorption band at 420 nm.

^{1}H NMR spectra were measured on a Bruker AM-400 spectrometer (400 MHz for ^{1}H NMR) and a Bruker CXP-300 spectrometer (300 MHz for ^{1}H NMR). Chemical shifts were referred to signals of an internal standard, DSS. MCD spectra were obtained on a JASCO J-500 spectropolarimeter equipped with a JASCO electromagnet which produces a longitudinal magnetic field up to 15.3 kG at the sample. Temperature of the sample was controlled with the stream of cold nitrogen gas evaporated from liquid nitrogen.

Results and Discussion

Paramagnetic Signals of ^{1}H NMR

Figures 1, A and B show low-field regions of 400 MHz ^{1}H NMR spectra of ferric low-spin P-450$_{scc}$ in the presence of aminoglutethimide and metyrapone, respectively. Broad signals were situated around 20, 28, 33 and 35 ppm for the aminoglutethimide solution and around 20 (2H) and 29 ppm for the metyrapone solution. Figures 1, C and D show the spectra of ferric low-spin P-450$_{scc}$ complexes caused by adding 20α-hydroxycholesterol and Tween 20. The 20α-hydroxycholesterol solution gave broad signals around 19, 25, 30 and 33 ppm, while the Tween 20 solution gave broad signals around 20, 23, 39 (2H) ppm. These NMR signals were temperature dependent and moved to the lower magnetic field as the temperature was decreased (Fig. 2). Thus, it was suggested [6, 7] that the signs correspond to a Curie-type behavior between 5 °C and 28 °C, that those signals are of paramagnetically shifted heme-methyl resonances and that those signals are ascribed to the dominant low-spin complexes. The paramagnetic shifts of the ferric low-spin P-450$_{scc}$ complexes were fairly divergent compared with those of the corresponding P-450$_{cam}$ complexes (Fig. 3). Thus, it was suggested that the heme environment of P-450$_{scc}$ is subtly different from that of P-450$_{cam}$.

It seemed likely that the second band (denoted by ○ in Figs. 1 and 3) from the high field reflects the axial ligand *trans* to the thiolate of P-450$_{scc}$. The low-spin P-450$_{scc}$ complexes caused by adding aminoglutethimide and metyrapone had the marker band around 28–29 ppm, while those by 20α-hydroxycholesterol and Tween 20 had the marker band around 25 ppm. The marker band will probably reflect the axial ligand *trans* to the thiolate anion in P-450$_{scc}$. It is suggested from MCD study [2] that low-spin P-450$_{scc}$ complexes bound with aminoglutethimide and metyrapone have nitrogenous axial 6th ligands, while those bound with 20α-hydroxycholesterol and Tween 20 have oxygenous 6th axial ligands. Thus, the nitrogen-bound form seems to have the band around 28–29 ppm, while the oxygen-bound form seems to have the band around 25 ppm. The same relationship is also observed for the P-450$_{cam}$ complexes, in that the lower-field signal of the CN–bound P-450$_{cam}$ is located at the position lower than that of the camphor-free P-450$_{cam}$ (oxygen-bound form) (Fig. 3) [6, 7].

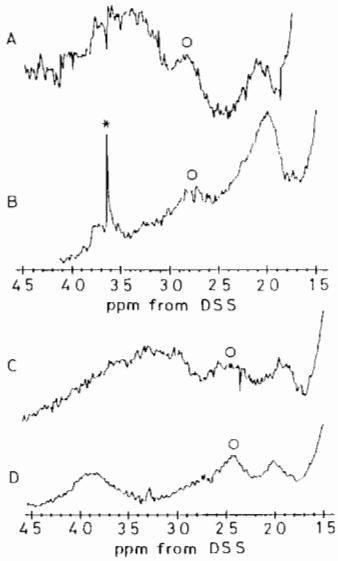

Fig. 1. 400 MHz ^{1}H NMR spectra of ferric low-spin P-450$_{scc}$ (0.3–0.5 mM) in the presence of aminoglutethimide (8 mM) (A), metyrapone (10 mM) (B), 20α-hydroxycholesterol (0.7 mM) (C) and Tween 20 (10 mM) (D). The solutions consisted of 99.9% D_2O, 50 mM potassium phosphate (pD 7.6), 0.1 mM EDTA and 0.1 mM dithiothreitol. The spectra were obtained at 28 °C. Asterisk denotes artificial noise. Other spectral conditions were: number of scans, 10 000; sweep width, 5000 Hz; exponential line broadenings, 50 Hz; pulse width, 14 μs (90° pulse); repetition time, 0.1 s; size, 8 k.

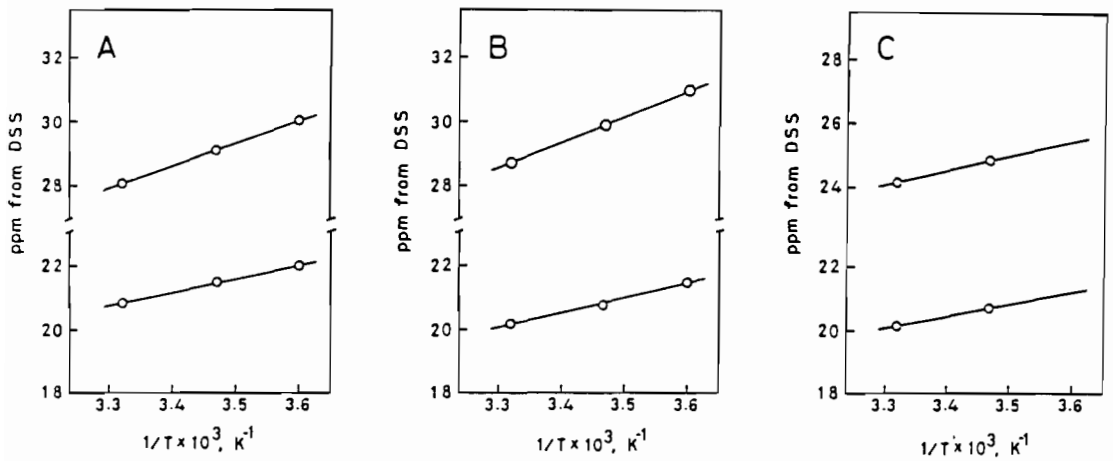

Fig. 2. Temperature dependences of 400 MHz ^1H NMR signals for ferric low-spin P-450$_{scc}$ (0.3–0.5 mM) in the presence of aminoglutethimide (8 mM) (A), metyrapone (10 mM) (B) and Tween 20 (10 mM) (C). The solutions consisted of 99.9% D_2O, 50 mM potassium phosphate (pD 7.6), 0.1 mM EDTA and 0.1 mM dithiothreitol. The ordinate shows chemical shifts in ppm from internal reference, DSS, while the abscissa shows the inverse of the absolute temperature. Due to dull signals of ferric low-spin P-450$_{scc}$ (0.3–0.5 mM) in the presence of 20α-hydroxycholesterol (0.7 mM), clear temperature dependence was not observed for this solution.

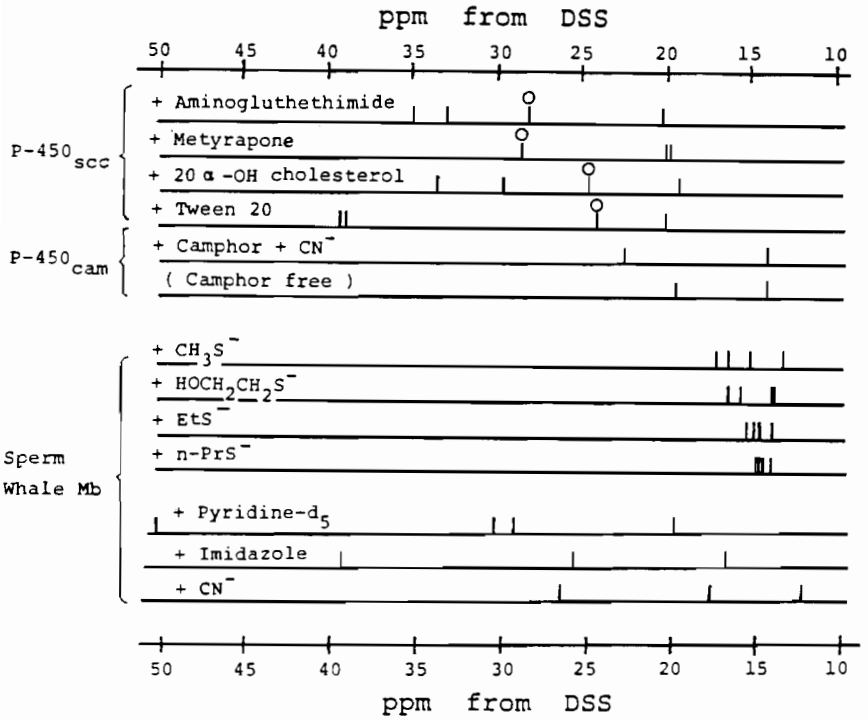

Fig. 3. Stick diagrams depicting paramagnetic shifts of ^1H NMR of various ferric low-spin P-450$_{scc}$, P-450$_{cam}$ and metMb complexes. Data for low-spin P-450$_{cam}$ complexes were taken from refs. 6 and 7. Spectra of P-450$_{scc}$ and metMb complexes were obtained at 28 °C and 35 °C, respectively. Spectra of CN^--bound and camphor-free forms of P-450$_{cam}$ were obtained at 15 °C and 30 °C, respectively [6, 7].

We obtained spectra of metMb–mercaptan complexes for reference. The signal positions for the metMb–mercaptan complexes were convergent to around 15 ppm, in contrast with those of the low-spin P-450$_{scc}$ and P-450$_{cam}$ complexes (Fig. 4). Thus, it was suggested that the electronic structures and/or the conformation of the internal thiolate ligand in P-450$_{scc}$ and P-450$_{cam}$ are different from those of the external thiolate-ligand in metMb–mercaptan complexes. Functional groups of distal amino acids in P-450 complexes may influence these characteristics. The signal convergence for the metMb–

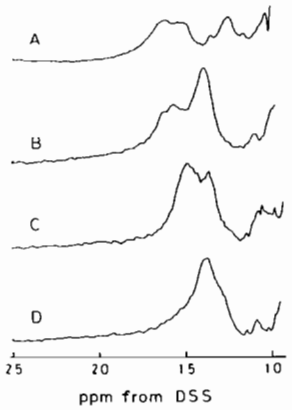

Fig. 4. 300 MHz ^{1}H NMR spectra of low-spin metMb (3 mM) complexes in the presence of 50–70 mM of methylmercaptan (A), mercaptoethanol (B), ethylmercaptan (C) and n-propylmercaptan (D). The solutions consisted of 99.9% D_2O and 50 mM potassium phosphate (pD 7.6). The spectra were obtained at 35 °C.

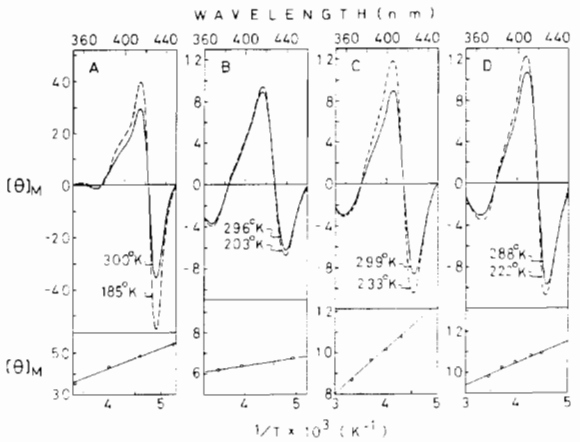

Fig. 5. Temperature dependence of MCD spectra at the Soret region for selected low-spin complexes: (A), metMb (15.5 μM)-KCN (6 mM); (B), metMb (32.3 μM)-n-propylmercaptan (8 mM); (C), P-450$_{scc}$ (33.4 μM)-20α-hydroxycholesterol (0.7 mM); (D), P-450$_{scc}$ (23.8 μM)-metyrapone (8 mM). The solutions of metMb consisted of 50% glycerol and 0.1 M potassium phosphate (pH 7.25) while the solutions of P-450$_{scc}$ consisted of 50% glycerol, 0.1 M potassium phosphate (pH 7.25), 0.1 mM EDTA and 0.1 mM dithiothreitol. Cell length, 2 mm or 3 mm; time constant, 4 s; band width, 2 nm; scan speed, 10 nm/min; sensitivity, 2 m°/cm.

mercaptan complexes seems likely to be dependent on the alkyl group of the mercaptans. The electron donating factor of the alkyl group in metMb–mercaptan complexes markedly influences the paramagnetic shifts of these complexes. We also noted that the NMR line-widths of the thiolate-ligated low-spin complexes (P-450 and metMb–mercaptan) are much larger than those of the non-thiolate-ligated low-spin complexes (metMb–CN^-, pyridine and imidazole).

Temperature Dependence of the Soret MCD

The temperature dependent S-shaped MCD band is called a C-term, which is caused by an electronic transition from a degenerated ground state to a non-degenerated excited state and is closely correlated with the Boltzmann distribution of the electron at the ground state. The Soret MCD band of the ferric low-spin heme complex has the S-shaped MCD band and has been known to be the C-term [12]. However, since it seemed likely that the Soret MCD spectra of ferric low-spin P-450 are peculiar in comparison with those of the corresponding low-spin metMb complexes [11], we examined the temperature dependence of the MCD spectra of P-450$_{scc}$, P-420$_{scc}$, rabbit liver P-450 complexes and metMb complexes (Fig. 5) (Table I). Temperature dependence was studied on the MCD through at the Soret region for the various ferric low-spin complexes. Since the MCD peak at the Soret region is composed of the π–π^* transition of porphyrin and a charge transfer band between Fe and porphyrin [12, 13], we did not use the MCD peak for evaluation of temperature dependence. The temperature dependences for thiolate-ligated hemoprotein complexes such as metMb–mercaptan and P-450 complexes were decidely lower than those observed for other nitrogen or cyanide-ligated hemoprotein complexes such as metMb–imidazole or metMb–CN. Since the temperature dependences for the P-450 complexes were more marked than those for the metMb–thiolate complexes, it is suggested here again that the electronic characters and/or the conformation of the internal thiolate ligand in P-450 complexes are different from those of the external thiolate ligand in metMb–thiolate complexes as suggested from ^{1}H NMR findings. The Soret MCD bands for probably oxygen-bound forms such as 20α-hydroxycholesterol and Tween 20–P-450$_{scc}$ complexes were relatively more temperature-sensitive than those for nitrogen-bound forms such as aminoglutethimide and metyrapone–P-450$_{scc}$ complexes.

The temperature dependences of P-450$_1$ and the P-448$_1$–CN^- complexes were in the category of non-nitrogenous 6th ligand, which is in accordance with the fact that low-spin type P-450$_1$ should have oxygenous 6th ligand [2–5]. It seemed likely, on the basis of the temperature dependence study, that P-420$_{scc}$ must have axial ligands other than the thiolate anion.

The absolute intensity of the Soret MCD band for the ferric low-spin hemoprotein does not seem to be related to the axial ligand because the metMb–N_3^- complex, which has relatively small Soret MCD band comparable to P-450 complexes and metMb–thiolate complexes, shows a remarkably temperature-dependent Soret MCD band [12].

TABLE I. Temperature Dependence of MCD Bands at the Soret Region for Various Low-spin Complexes.

Complexes	Temperature dependence $\Delta[\theta]_M/\Delta(1/T)$	Internal ligand[a] (left) external ligand (right)
metMb–CN^-	9.3	N–Fe–C
metMb–Imidazole	5.1	N–Fe–N
metMb–SCH_3	0.5	N–Fe–S
metMb–SCH_2CH_3	0.6	N–Fe–S
metMb–$SCH_2CH_2CH_3$	0.4	N–Fe–S
P-420$_{scc}$[b]	11.4	?–Fe–?
P-450$_{scc}$–20α-OHChol[c]	2.2	S–Fe–O
P-450$_{scc}$–Tween 20	1.3	S–Fe–O
P-450$_{scc}$–Aminoglu[d]	1.2	S–Fe–N
P-450$_{scc}$–Metyrapone	1.1	S–Fe–N
P-450$_1$	2.6	S–Fe–O
P-448$_1$–CN^-	2.3	S–Fe–C

[a]Internal ligand of metMb is imidazole of His, while that of P-450 is probably thiolate of Cys-355 for P-450$_{cam}$, Cys-461 for P-450$_{scc}$ or Cys-436 for P-450$_{b,e}$ (*cf.* ref. 11). [b]*cf.* Experimental. [c]20α-hydroxycholesterol. [d]Aminoglutethimide.

Conclusion

(1) The paramagnetic shifts for ferric low-spin P-450$_{scc}$ were divergent compared with those for corresponding P-450$_{cam}$, suggesting that there is subtle difference in the heme environment between P-450$_{scc}$ and P-450$_{cam}$. (2) The ferric low-spin P-450$_{scc}$ complexes formed by adding nitrogenous ligands such as metyrapone and aminoglutethimide have marker signals of ^1H NMR which are different from those formed by adding 20α-hydroxycholesterol and Tween 20. (3) The paramagnetic shifts for metMb–mercaptan complexes are convergent compared with those for ferric low-spin P-450$_{scc}$ and P-450$_{cam}$, suggesting that the electronic character and/or the conformation of the internal thiolate ligand in P-450$_{scc}$ and P-450$_{cam}$ are different from those of the external thiolate ligand in metMb–thiolate complexes. (4) The electron donating factor of the alkyl group of the mercaptans bound with metMb markedly influences the ^1H NMR paramagnetic shifts of the metMb–thiolate complexes. (5) A thiolate ligation to the ferric heme iron markedly reduced the temperature-dependence of the Soret MCD band for the ferric low-spin heme complexes. (6) The conclusion (3) was reconfirmed by the temperature dependence of the Soret MCD bands for various P-450 complexes and metMb–thiolate complexes. (7) Whether a ligand *trans* to a thiolate ligand in ferric low-spin P-450 is nitrogen or non-nitrogen was differentiated in terms of the temperature dependence of the Soret MCD bands.

Therefore, it should be emphasized that ferric low-spin heme complexes actually take various electronic states depending on the axial ligands or the distal amino acids in the protein.

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