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Electron Paramagnetic Resonance Study of Ferrous Cytochrome P-450$_{scc}$–Nitric Oxide Complexes: Effects of Cholesterol and Its Analogues†

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ABSTRACT: Electron paramagnetic resonance (EPR) spectra of nitric oxide (NO) complexes of ferrous cytochrome P-450$_{scc}$ were measured at 77 K for the first time without using the rapid-mixing and freeze-quenching technique. Without substrate the EPR spectra were very similar to those of cytochrome P-450$_{cam}$ (from *Pseudomonas putida*) and cytochrome P-450$_{LM}$ (from rat liver microsomes) with rhombic symmetry; $g_x = 2.071$, $g_z = 2.001$, $g_y = 1.962$, and $A_z = 2.2$ mT for ^{14}NO complexes. Upon addition of substrates [such as cholesterol, 22(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, 25-hydroxycholesterol, and 22-ketocholesterol], the EPR spectra exhibited many variations having rhombic symmetry in the major component and an additional minor component with less rhombic symmetry. Furthermore, addition of 20(*S*)-hydroxycholesterol caused a striking change in the EPR spectrum. The component with rhombic symmetry disappeared completely, and the component with less rhombic symmetry dominated ($g_x = 2.027$, $g_z = 2.007$, $g_y = 1.984$, and $A_z = 1.76$ mT for ^{14}NO complexes). These observations suggest the existence of the following physiologically important natures: (1) the conformational flexibility of the active site of the enzyme due to the steric interaction between the substrate and the heme-bound ligand molecule and (2) the importance of the hydroxylation of the cholesterol side chain at the 20*S* position to proceed the side-chain cleavage reaction in cytochrome P-450$_{scc}$.

Cytochrome P-450$_{scc}$,[1] which is located in the inner mitochondrial membrane of the adrenal cortex, catalyzes the cholesterol side-chain cleavage reaction. The cholesterol side-chain cleavage reaction involves three consecutive hydroxylation steps. The first hydroxylation occurs at the 22*R* position to yield 22(*R*)-hydroxycholesterol, the second occurs at the 20*S* position to give 20(*R*),22(*R*)-dihydroxycholesterol, and the third oxidative cleavage of the C20–C22 bond of the diol results in a formation of pregnenolone and isocaproic aldehyde (Burstein et al., 1975; Burstein & Gut, 1976; Hume et al., 1984). The successive conversion of cholesterol to pregnenolone is ensured by increased affinity of the hydroxylated intermediates to the enzyme and by increased stability of the ferrous dioxygen complex in each step (Tuckey & Kamin, 1982, 1983). These observations suggest the existence of strict stereochemical interactions among cholesterol side-chain, heme-bound dioxygen, and surrounding amino acid residues.

In order to elucidate the mechanism of the cytochrome P-450$_{scc}$ mediated side-chain cleavage reaction, it is necessary to have a detailed description of its active site. Since nitric oxide (NO) contains one unpaired electron, NO has been used as a paramagnetic probe of the ferrous–heme moiety in a variety of hemoproteins, particularly as a model for dioxygen-binding hemoproteins (Yonetani et al., 1972).

Several preliminary reports have suggested that nitric oxide combines with the microsomal cytochrome P-450 (Ullrich et al., 1968; Miyake et al., 1968). The observed EPR spectra of the ferrous–NO complexes prepared by NO-reduced and dithionite-reduced enzymes (Miyake et al., 1968, 1969), however, were very similar to the denatured ferrous–NO complex of hemoglobin (Kon, 1968, 1975). Ebel et al. (1975) found that the ferrous microsomal cytochrome P-450–NO complex was very labile and was converted quickly to a species which had an EPR spectrum similar to that of ferrous P-420–NO and/or denatured ferrous–NO complex of hemoglobin. Therefore, they had to measure the EPR spectra of the ferrous–NO complexes of cytochrome P-450$_{LM}$ and cytochrome P-450$_{cam}$ by rapidly mixing the dithionite-reduced

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[1] Abbreviations: EPR, electron paramagnetic resonance; cytochrome P-450$_{scc}$, cytochrome P-450 present in inner mitochondrial membranes of bovine adrenal cortex, which catalyzes the cholesterol side-chain cleavage reaction; cytochrome P-450$_{cam}$, cytochrome P-450 obtained from *Pseudomonas putida* grown on *d*-camphor; cytochrome P-450$_{LM}$, cytochrome P-450 present in microsomes isolated from livers of phenobarbital-treated male albino rats; EDTA, ethylenediaminetetraacetic acid.

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enzymes with NO-saturated buffer followed by freeze-quenching within 15 ms. Using this rapid-mixing and freeze-quenching technique, O'Keefe et al. (1978) found that the EPR spectra of NO complexes of ferrous cytochrome P-450 had rhombic symmetry with a triplet hyperfine splitting in the g_z (or g_3) signal. Unlike horseradish peroxidase, there was no nine-lined superhyperfine structure for the g_z signal, consistent with the proposal of a cysteinyl thiolate as the axial ligand bound trans to the nitric oxide binding site because sulfur possesses no nucleus spin. This proposal was recently confirmed by X-ray crystallographic analysis of cytochrome P-450_{cam} (Poulos et al., 1985).

In the present study, we have found that the nitric oxide complex of cytochrome P-450_{scc} is very stable, particularly in the presence of substrate or its analogues, which has enabled us to measure the EPR spectra for the first time without using the rapid-mixing and freeze-quenching technique. The detailed analyses of present data have suggested the conformational flexibility of the active site of cytochrome P-450_{scc}, and the hydroxylation at position 20*S*, particularly, caused a drastic stereochemical change around the heme, which may, in turn, ensure the obligatory sequential hydroxylation steps of cholesterol side-chain cleavage. This conclusion is consistent with the result obtained from a resonance Raman study of carbon monoxide complexes of ferrous cytochrome P-450_{scc} in the presence of various hydroxylated cholesterols and analogues; a detailed description of the resonance Raman study can be found in the following paper in this issue (Tsubaki et al., 1987).

Experimental Procedures

Materials. Cholesterol, 22(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, 20(*S*)-hydroxycholesterol, and 22-ketocholesterol were purchased from Sigma; 25-hydroxycholesterol was obtained from Steraroids, Inc. Isotopically labeled $Na^{15}NO_2$ was obtained from Merck and Co. Other chemicals, including glycerol, NaCl, EDTA, and KOH, were obtained from Wako Pure Chemicals, Inc., and were used without further purification.

Preparation of Cytochrome P-450_{scc} Samples. Cytochrome P-450_{scc} from bovine adrenocortical mitochondria was purified as previously described (Tsubaki et al., 1986a). Endogenous cholesterol and its hydroxylated intermediate metabolites, if any, were removed during the sample purification because of the continuous use of a nonionic detergent, Emulgen 913, in the buffer system (Tsubaki et al., 1986b). Emulgen 913 was then removed by adrenodoxin–Sepharose 4B column chromatography as previously described (Tsubaki et al., 1986a). The obtained sample was practically free from Emulgen 913, as judged by the absorption spectra in the ultraviolet region, and was in pure low-spin form. Emulgen 913 depleted sample was dialyzed against 20 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 100 mM NaCl, and 0.1 mM EDTA. The purity of the sample at this stage was checked spectrophotometrically in ferrous carbon monoxide form (ϵ = 129.1 mM^{-1} cm^{-1} at 448 nm) (Tsubaki et al., 1986b). The specific heme content of the sample was more than 15 nmol of P-450/mg of protein and was practically free of the P-420 form.

Cholesterol, 22(*R*)-hydroxycholesterol, 22(*S*)-hydroxycholesterol, 20(*S*)-hydroxycholesterol, 25-hydroxycholesterol, and 22-ketocholesterol in methanol (10 mg each/1 mL) were added to about 1500 nmol of the dialysate (80–100 mL in volume) to a final steroid concentration of about 25–30 mM, assuming it fully dissolves, and incubated on ice for more than 24 h to ensure the complete formation of cytochrome P-$450_{scc}(Fe^{3+})$ substrate (or its analogue) complex; we assumed that equilibrium was attained. After the inspection by light absorption spectra of these complexes with a Shimadzu UV-240 spectrophotometer, the sample was concentrated by centrifugation at 3000 rpm using CENTRIFLO membrane cones (Type CF25, Amicon Corp.) to about 0.5–0.6 mM (final volume 2.5–3.0 mL), which was used directly for the EPR measurements.

Measurements of EPR Spectra. EPR measurements were carried out at X-band (9.35 GHz) microwave frequency by using a Varian X-band cavity with a home-built EPR spectrometer with 100-kHz field modulation. An immersion Dewar flask was used for measurements at 77 K, whereas a Varian variable-temperature Dewar was used for measurements above 77 K.

Before complexation with nitric oxide, parts of cytochrome P-450_{scc}–substrate (or its analogue) complexes in oxidized form were transferred into EPR sample tubes and were slowly immersed in liquid nitrogen (77 K) until frozen (15–30 s), and the EPR spectra of their low-spin component were measured to reconfirm the complete formation of substrate–enzyme complexes by comparison with the data reported previously (Orme-Johnson et al., 1979). Cytochrome P-450_{scc} samples in EPR sample tubes with screw-cap septums were deoxygenated by repeated evacuation and flushing of oxygen-free N_2 gas. Solid sodium dithionite was anaerobically introduced to reduce the oxidized form of cytochrome P-450_{scc} and, then, NO gas, previously washed with 1 M KOH, was introduced with an airtight syringe. The sample was kept on ice to complete the formation of the ferrous–NO complex. At appropriate intervals, the EPR spectrum was checked. For the experiments for isotopically labeled ^{15}NO, an appropriate amount of solid $Na^{15}NO_2$ was placed inside the EPR tube before deoxygenation of the sample solution, and then solid sodium dithionite was added anaerobically to form the ferrous–^{15}NO complex.

Results

Oxidized States. Cytochrome P-450_{scc} as isolated, cytochrome P-450_{scc} (–Chol) [a minus (–) sign indicates the absence of substrate], was in pure ferric low-spin form as judged by visible absorption spectrum at room temperature (25 °C) (Tsubaki et al., 1986a). The EPR spectrum of the oxidized cytochrome P-450_{scc} (–Chol) at 77 K showed characteristic features at g_z = 2.419, g_y = 2.243, and g_x = 1.913 (Figure 1). There was no indication of high-spin (EPR) signal. Upon addition of various steroids [cholesterol, 22(*R*)-hydroxycholesterol, 22(*S*)-hydroxycholesterol, 20(*S*)-hydroxycholesterol, 25-hydroxycholesterol, and 22-ketocholesterol] to the oxidized cytochrome P-450_{scc}(–Chol), the EPR spectra showed a variety of changes in spin state; cholesterol and 25-hydroxycholesterol produced heme species that were predominantly in high spin, while 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, 22(*S*)-hydroxycholesterol, and 22-ketocholesterol produced heme species that were predominantly in low spin, as has been reported previously by Orme-Johnson et al. (1979). Since high-spin EPR signals of cytochrome P-450_{scc} (g = 8, 3.68, and 1.8) were only observed at 4.2 K and prolonged signal averagings were needed at 77 K to obtain high-spin EPR spectra in good quality, we observed only low-spin signals as a measure for substrate–enzyme complex formation. As shown in Figure 1, our results were essentially consistent with those obtained by Orme-Johnson et al. (1979). The 22(*R*)-hydroxycholesterol complex showed the largest g_z value (2.463), whereas the 20(*S*)-hydroxycholesterol complex showed the smallest g_z value (2.400). Other steroid complexes had intermediate g_z values between these two.

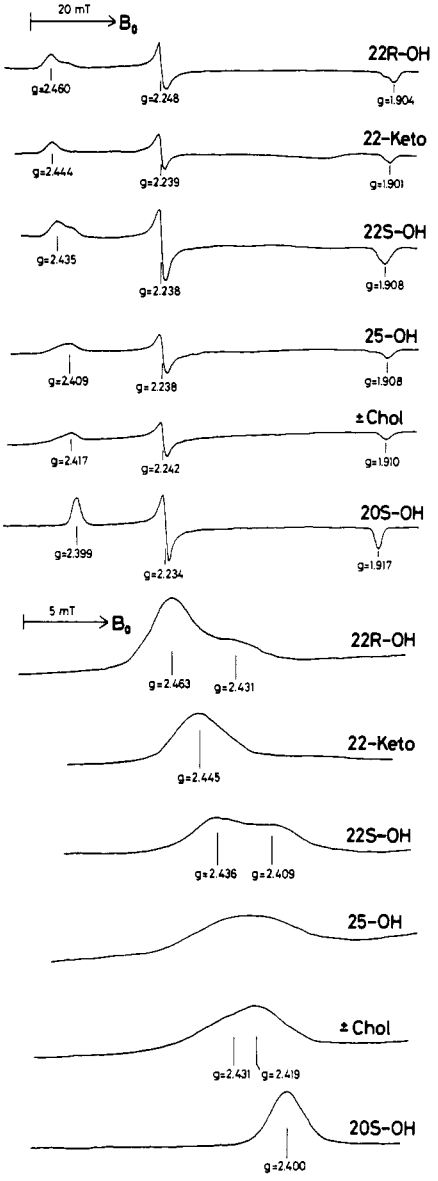

FIGURE 1: (Top) EPR spectra of ferric low-spin cytochrome P-450$_{scc}$–steroid complexes. Conditions of EPR spectroscopy: microwave frequency, 9.35 GHz (X-band); microwave power, 5 mW; modulation frequency, 100 kHz; amplitude, 0.3 mT; temperature, 77 K. (Bottom) EPR spectra of ferric low-spin cytochrome P-450$_{scc}$–steroid complexes in g_z region. The conditions of EPR spectroscopy were the same as before except that the scale of the magnetic field was expanded by 4 times. Abbreviations: 22R-OH, 22(*R*)-hydroxycholesterol; 22-Keto, 22-ketocholesterol; 22S-OH, 22(*S*)-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; Chol, cholesterol; 20S-OH, 20(*S*)-hydroxycholesterol.

Ferric low-spin signals of cytochrome P-450$_{scc}$ (–Chol) were heterogeneous as shown by a shoulder appearing in lower field region of the g_z signal (g = 2.419) of the major species (Figure 1); the existence of this minor species was reported previously by Orme-Johnson et al. (1979). They estimated that this minor form accounted for not more than 10% of the total P-450 concentration and did not bind to 20(*S*)-hydroxycholesterol, 22(*S*)-hydroxycholesterol, or 20(*R*),22(*R*)-dihydroxycholesterol (in other words, they considered that this minor form corresponded to the denatured form of cytochrome P-450$_{scc}$). In the present study, addition of cholesterol to cytochrome P-450$_{scc}$ (–Chol) caused a significant decrease of the total population of ferric low-spin species. But the relative population of this minor form in ferric low-spin species seemd to remain the same, since the shapes of EPR spectra of ferric low-spin species from cytochrome P-450$_{scc}$ with and without cholesterol were identical (Figure 1). Therefore, we thought this minor form represented a conformer of the ferric low-spin state. Heterogeneity in ferric low-spin species were observed for all other steroid complexes except for the 20(*S*)-hydroxycholesterol complex (Figure 1).

Nitric Oxide Complex of Ferrous Cytochrome P-450$_{scc}$ in the Absence of Substrate. The ^{14}NO complex of ferrous cytochrome P-450$_{scc}$ in the absence of substrate exhibited EPR signals centered around g = 2 having rhombic symmetry at 77 K (Figure 2). The absorption at g = 2.001 has a well-resolved triplet hyperfine splitting with 2.16 mT. The EPR spectra of ^{14}NO and ^{15}NO complexes of ferrous cytochrome P-450$_{scc}$ (–Chol) were very similar to those of ^{14}NO and ^{15}NO complexes of ferrous cytochrome P-450$_{cam}$ (±camphor) and of ^{14}NO complex of ferrous cytochrome P-450$_{LM}$ (+substrate) obtained from liver microsomes of male rats (O'Keefe et al., 1978), and similar EPR spectra have been observed with ferrous–NO complexes of horseraish peroxidase (Yonetani et al., 1972), chloroperoxidase (Chiang et al., 1975), L-tryptophan 2,3-dioxygenase (Henry et al., 1976), lactoperoxidase (Yonetani et al., 1972), and to a lesser extent catalase (Yonetani et al., 1975) (see Table I for comparison).

The most prominent difference between cytochrome P-450$_{scc}$ and cytochrome P-450$_{cam}$ or cytochrome P-450$_{LM}$ was the stability of the ferrous–NO complex. The NO complex of ferrous cytochrome P-450$_{LM}$ was especially unstable. O'Keefe et al. (1978) had to measure the EPR spectra of the NO complex of ferrous cytochrome P-450$_{LM}$ prepared by rapidly mixing the dithionite-reduced enzyme with NO-saturated buffer and freeze-quenching within 15 ms. On the other hand, our NO complex of ferrous cytochrome P-450$_{scc}$ was extremely stable for at least several hours on ice, although a slight amount of formation of the P-420 form (see below) was inevitable during the reduction of the heme as evidenced in Figure 2. The amount of this NO complex of the ferrous P-420 form, however, did not increase any more.

NO Complex of Ferrous P-420 Form. The EPR spectra of the NO complex of the ferrous P-420 form arising from the denaturation of ferrous–NO complex of cytochrome P-450$_{scc}$ by alkaline treatment or standing at room temperature for a longer time were essentially identical with those of NO complexes of ferrous P-420$_{LM}$ and ferrous P-420$_{cam}$ as shown in Figure 3 (Ebel et al., 1975; O'Keefe et al., 1978). The EPR spectra were characterized by sharp triplet (for ^{14}NO) and doublet (for ^{15}NO) splittings of the g_z signal with small hyperfine constants (1.67 and 2.4 mT, respectively). The g_x and g_y values could not be determined accurately due to spectral uncertainty (see Table I). Kon (1975) has shown that such hyperfine splitting in 1.6–1.8 mT (for ^{14}NO) will be observed for five-coordinated heme model NO complexes, whereas six-coordinated heme model NO complexes give hyperfine splitting constants of 2.0–2.3 mT (for ^{14}NO). Thus, upon denaturation of NO complex of ferrous P-450 to NO complex of ferrous P-420, the transaxial ligand (cysteinyl thiolate) to the heme is removed or the bond may be severely distorted, as suggested by O'Keefe et al. (1978).

Effects of Cholesterol and Its Analogues on Ferrous–NO Complex. O'Keefe et al. (1978) reported that addition of exogeneous substrate, such as hexobarbital, stabilized the NO complex of ferrous cytochrome P-450$_{LM}$, although the stabilization was only on the order of several minutes of longer lifetime compared to that without substrate. We examined the effects of cholesterol and its analogues on the EPR spectrum of NO complexes of ferrous cytochrome P-450$_{scc}$. Addition of cholesterol prior to the ferrous–NO complex for-

Table I: Electron Paramagnetic Parameters of Nitric Oxide Complexes of Ferrous Hemoproteins

complex[a]	principal g value			hyperfine coupling constant A_z (mT)	ref
	g_x	g_z	g_y		
P-450$_{scc}$–^{14}NO (–Chol)	2.071	2.001	1.962	2.20	this study
P-450$_{cam}$–^{14}NO (±camphor)	2.073	2.009	1.976	1.92	O'Keefe et al. (1978)
P-450$_{LM}$–^{14}NO (+substrate)	2.068	2.008	1.978	2.0	O'Keefe et al. (1978)
HRP–^{14}NO	2.080	2.004	1.955	2.05	Yonetani et al. (1972)
HRP–^{14}NO	2.076	2.008	1.967	2.08	O'Keefe et al. (1978)
CCP–^{14}NO	2.080	2.004	1.960	2.1	Yonetani et al. (1972)
CPO–^{14}NO	2.082	2.004	1.975	2.0	Chiang et al. (1975)
TDO–^{14}NO (–L-Trp)	2.094	2.009	1.988	1.65	Henry et al. (1976)
TDO–^{14}NO (+L-Trp)	2.088	2.004	1.982	2.0	Henry et al. (1976)
LPO–^{14}NO	2.070	2.004	1.958	1.6	Yonetani et al. (1972)
catalase–^{14}NO	2.050	2.003	1.970	2.1	Yonetani et al. (1972)
P-450$_{scc}$–^{15}NO (–Chol)	2.070	2.000	1.962	3.05	this study
P-450$_{cam}$–^{15}NO (±camphor)	2.074	2.009	1.975	2.74	O'Keefe et al. (1978)
HRP–^{15}NO	2.080	2.004	1.955	3.0	Yonetani et al. (1972)
HRP–^{15}NO	2.076	2.008	1.966	3.0	O'Keefe et al. (1978)
CPO–^{15}NO	2.082	2.004	1.975	2.8	Chiang et al. (1975)
P-420$_{scc}$–^{14}NO		2.007		1.67	this study
P-420$_{cam}$–^{14}NO	2.072	2.015	2.072	1.6	O'Keefe et al. (1978)
P-420$_{LM}$–^{14}NO	2.074	2.015	2.074	1.6	O'Keefe et al. (1978)
P-420$_{scc}$–^{15}NO		2.009		2.40	this study
P-420$_{LM}$–^{15}NO	2.072	2.015	2.072	2.2	O'Keefe et al. (1978)

[a] Abbreviations: P-450$_{scc}$, cytochrome P-450$_{scc}$; P-450$_{cam}$, cytochrome P-450$_{cam}$; P-450$_{LM}$, cytochrome P-450$_{LM}$; HRP, horseradish peroxidase; CCP, cytochrome c peroxidase; CPO, chloroperoxidase; TDO, L-tryptophan 2,3-dioxygenase; LPO, lactoperoxidase.

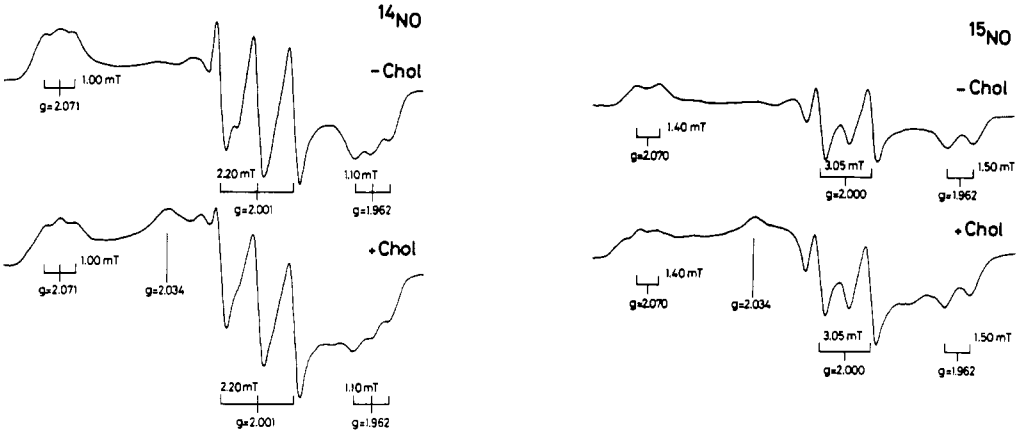

FIGURE 2: (Left) EPR spectra of ^{14}NO complex of ferrous cytochrome P-450$_{scc}$ in the absence (–Chol) and in the presence (+Chol) of cholesterol. (Right) EPR spectra of ^{15}NO complex of ferrous cytochrome P-450$_{scc}$ in the absence (–Chol) and in the presence (+Chol) of cholesterol. The conditions of EPR spectroscopy were the same as in Figure 1.

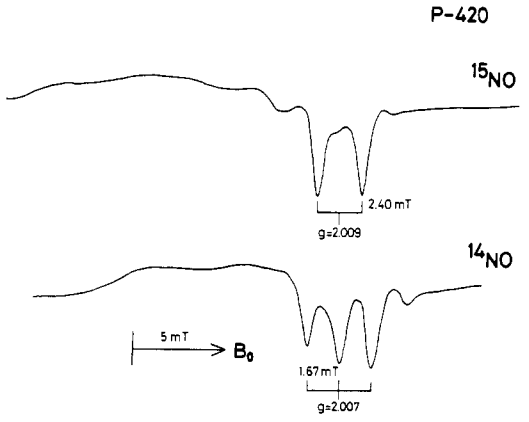

FIGURE 3: EPR spectra of ^{15}NO (upper spectrum) and ^{14}NO (lower spectrum) complexes of cytochrome P-450$_{scc}$. The conditions of EPR spectroscopy were the same as in Figure 1.

mation caused no significant change on the EPR spectrum, except for a small amount of formation of a new species as evidenced by g = 2.03 signal (Figure 2). The presence of cholesterol apparently stabilized the NO complex of ferrous cytochrome P-450$_{scc}$ compared to the complex without substrate; even after overnight incubation on ice, practically no increase in the P-420 form was observed.

Addition of 25-hydroxycholesterol or 22(S)-hydroxycholesterol prior to ferrous–NO complex formation yielded a very similar effect on the EPR spectrum to that of cholesterol, similar EPR spectra with rhombic symmetry (g_x = 2.067–2.074, g_z = 1.998–2.000, g_y = 1.960–1.962, A_z = 2.91–3.05 mT for ^{15}NO and A_z = 2.1–2.2 mT for ^{14}NO), and formation of the new ferrous NO species with less rhombic symmetry indicated by g = 2.03 signals (Figure 4).

Addition of 22-ketocholesterol caused a slight decrease of anisotropy [the g_x value decreased to 2.060 (2.058), whereas the g_y value increased to 1.968 (1.967) for ^{15}NO (^{14}NO)] with almost the same hyperfine splitting constants as those of cholesterol, 25-hydroxycholesterol, and 22(S)-hydroxycholesterol complexes. The minor component of ferrous–NO complex at g = 2.03 still existed (Figure 4).

20(S)-Hydroxycholesterol caused a most striking effect on the spectrum in the present study: a dramatic decrease of anisotropy [the g_x value decreased to 2.029 (2.027), whereas the g_y value increased to 1.986 (1.984) for ^{15}NO (^{14}NO)] and of hyperfine splitting constants [A_z = 1.76 and 2.33 mT for ^{14}NO and ^{15}NO, respectively)]. This type of EPR spectrum

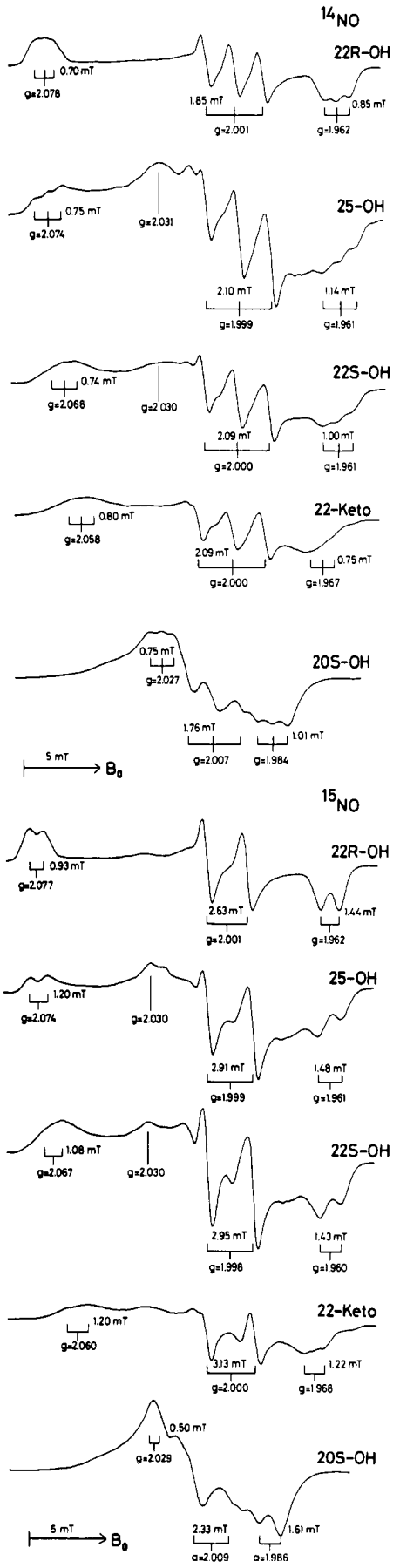

FIGURE 4: (Top) EPR spectra of ^{14}NO complexes of ferrous cytochrome P-450$_{scc}$ in the presence of various steroids. (Bottom) EPR spectra of ^{15}NO complexes of cytochrome P-450$_{scc}$ in the presence of various steroids. The conditions of EPR spectroscopy were the same as in Figure 1. The abbreviations used are the same as in Figure 1.

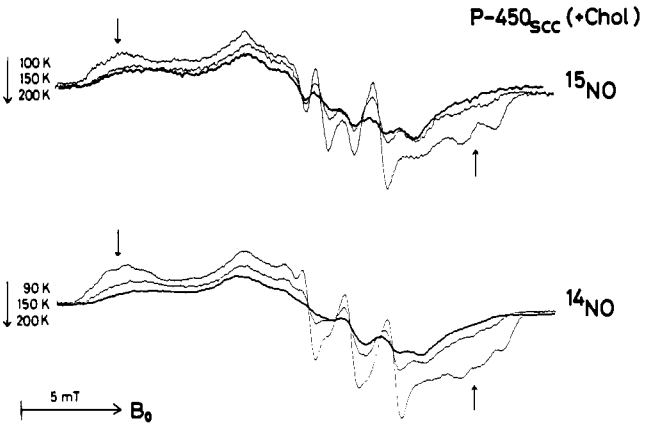

FIGURE 5: Temperature dependence of EPR spectra of ^{15}NO (upper) and ^{14}NO (lower) complexes of ferrous cytochrome P-450$_{scc}$ in the presence of cholesterol. The temperature was increased from 77 to 200 K, and only several spectra in the intermediate temperature as indicated in the figure are shown. Other conditions for EPR spectroscopy were the same as in Figure 1.

has never before been reported for NO complexes of ferrous cytochrome P-450 (Figure 4).

On the other hand, addition of 22(R)-hydroxycholesterol caused an opposite effect: an increase of g anisotropy [the g_x value showed a shift to 2.077 (2.078) for ^{14}NO (^{15}NO) but the g_y value remained almost the same] and a lowering of hyperfine splitting constants to A_z = 1.85 and 2.63 mT for ^{14}NO and ^{15}NO, respectively. In addition, the EPR signal at g = 2.03 disappeared completely (Figure 4). These results were summarized in Table II.

Temperature Dependence of EPR Spectra for Ferrous–NO Complexes. The temperature dependence of the EPR spectra for the NO complex of ferrous cytochrome P-450$_{scc}$ was investigated in the presence of cholesterol (for ^{14}NO and ^{15}NO), 22(R)-hydroxycholesterol (for ^{15}NO), and 20(S)-hydroxycholesterol (for ^{15}NO). As the temperature increased, the intensity of the rhombic EPR absorption decreased rapidly, but the shape of the EPR spectrum for the 20(S)-hydroxycholesterol complex was essentially independent of the temperature in the range between 77 and 200 K, whereas the spectrum for the 22(R)-hydroxycholesterol complex showed broadening upon warming. However, their g values seemed to remain the same.

The effect of the temperature was most significant for the cholesterol complex as shown in Figure 5. The EPR absorption of the major component with rhombic symmetry became broader with decreasing intensity, just as in the case of the 22(R)-hydroxycholesterol complex upon warming, whereas the minor component with less rhombic symmetry became dominant in the spectra. The dominant component with less rhombic symmetry at 200 K was very similar to that of the 20(S)-hydroxycholesterol complex. This observation confirmed our proposal that the minor components with less rhombic symmetry in the EPR spectra of NO complexes of ferrous cytochrome P-450$_{scc}$ in the presence of cholesterol, 20(S)-hydroxycholesterol, and 25-hydroxycholesterol were similar species to that in the presence of 20(S)-hydroxycholesterol.

Discussion

Stability of Ferrous–NO Complex. The binding of cholesterol, hydroxycholesterols, and 22-ketocholesterol to cytochrome P-450$_{scc}$ caused a significant increase in the stability of the ferrous–NO complex (at 0 °C) relative to that without substrate, although no quantitation was made. Our present observation is comparable to the 15-fold increase in half-time

Table II: Effect of Steroids on Electron Paramagnetic Parameters of Nitric Oxide Complexes of Ferrous Cytochrome P-450$_{scc}$

steroid	^{15}NO		^{14}NO	
	g value	hyperfine coupling constant[a] (mT)	g value	hyperfine coupling constant[a] (mT)
substrate free (−Chol)	g_x = 2.070 g_z = 2.000 g_y = 1.962	A_x = 1.40 A_z = 3.05 A_y = 1.50 (24.9%)	g_x = 2.071 g_z = 2.001 g_y = 1.962	A_x = 1.00 A_z = 2.20 A_y = 1.10 (25.2%)
+cholesterol[b]	g_x = 2.070 g_z = 2.000 g_y = 1.962	A_x = 1.40 A_z = 3.05 A_y = 1.50 (24.9%)	g_x = 2.071 g_z = 2.001 g_y = 1.962	A_x = 1.00 A_z = 2.20 A_y = 1.10 (25.2%)
+22-ketocholesterol[b]	g_x = 2.060 g_z = 2.000 g_y = 1.968	A_x = 1.20 A_z = 3.13 A_y = 1.22 (29.1%)	g_x = 2.058 g_z = 2.000 g_y = 1.967	A_x = 0.80 A_z = 2.09 A_y = 0.75 (28.0%)
+22(S)-hydroxycholesterol[b]	g_x = 2.067 g_z = 1.998 g_y = 1.960	A_x = 1.08 A_z = 2.95 A_y = 1.43 (25.9%)	g_x = 2.068 g_z = 2.000 g_y = 1.961	A_x = 0.74 A_z = 2.09 A_y = 1.00 (26.1%)
+25-hydroxycholesterol[b]	g_x = 2.074 g_z = 1.999 g_y = 1.961	A_x = 1.20 A_z = 2.91 A_y = 1.48 (24.3%)	g_x = 2.074 g_z = 1.999 g_y = 1.961	A_x = 0.75 A_z = 2.10 A_y = 1.14 (25.0%)
+22(R)-hydroxycholesterol	g_x = 2.077 g_z = 2.001 g_y = 1.962	A_x = 0.93 A_z = 2.63 A_y = 1.44 (22.2%)	g_x = 2.078 g_z = 2.001 g_y = 1.962	A_x = 0.70 A_z = 1.85 A_y = 0.85 (23.2%)
+20(S)-hydroxycholesterol	g_x = 2.029 g_z = 2.009 g_y = 1.986	A_x = 0.50 A_z = 2.33 A_y = 1.61 (19.6%)	g_x = 2.027 g_z = 2.007 g_y = 1.984	A_x = 0.75 A_z = 1.76 A_y = 1.01 (19.4%)

[a] The numbers in parentheses are the spin densities of the nitrogen atom of nitric oxide, estimated by the method of McNeil et al. (1965). [b] Parameters only for the major components are summarized in this table.

for autoxidation of the ferrous–dioxygen complex of cytochrome P-450$_{scc}$ when cholesterol is bound (Tuckey & Kamin, 1982) and to the 12-fold increase in half-time for cytochrome P-450$_{cam}$ when camphor is bound (Eisenstein et al., 1977). Moreover, the binding of the hydroxycholesterol intermediates to cytochrome P-450$_{scc}$ caused a further 3–17-fold increase in the stability of the ferrous dioxygen complex in the order 20(R),22(R)-dihydroxycholesterol > 22(R)-hydroxycholesterol > 20(S)-hydroxycholesterol > cholesterol (Tuckey & Kamin, 1982), but the rate of the autoxidation for the dioxygen complex of ferrous cytochrome P-450$_{scc}$ in the presence of 22-ketocholesterol was similar to that in the presence of cholesterol (Tuckey & Kamin, 1982). These observations may suggest a specific interaction, such as hydrogen bonding, between the 22(R)-hydroxyl group and heme-bound dioxygen. This suggestion was recently made by Heyl et al. (1986). They claimed that the 22(R)-hydroxyl group localized on a line perpendicular to the heme plane, between 2 and 3 Å from the iron on the basis of specific interference of the binding of 22(R)-hydroxy steroids by heme-bound carbon monoxide (but not dioxygen). This conclusion is consistent with our recent resonance Raman study of cytochrome P-450$_{scc}$ (Tsubaki et al., following paper in this issue). It is not clear at present that the same type of interaction is operative in the NO complex of ferrous cytochrome P-450$_{scc}$.

Interactions between Ferrous–NO Moiety and Side-Chain Group. The EPR spectra of the NO complexes of ferrous cytochrome P-450$_{scc}$ in the presence of (or absence of) cholesterol, 22-ketocholesterol, and the hydroxycholesterols, except for 20(S)-hydroxycholesterol, exhibited well-defined g values with rhombic symmetry and hyperfine structure due to the NO nitrogen similar to those of NO complexes of ferrous horseradish peroxidase and cytochrome c peroxidase (Yonetani et al., 1972). In contrast, the EPR spectra of the NO complexes of ferrous cytochrome P-450$_{scc}$ in the presence of 20-(S)-hydroxycholesterol exhibited less rhombic symmetry with slightly smaller hyperfine splitting constants compared to the others.

It is well-known that the unpaired electron of nitric oxide of NO complexes of ferrous hemoglobin or horseradish peroxidase is considerably delocalized to the heme iron (only 20–40% of the spin density remained in the nitrogen atom of NO in these hemoproteins) (Kon & Kataoka, 1969; Yonetani et al., 1972). Thus almost the same situation was expected for the NO complex of ferrous cytochrome P-450$_{scc}$. We could estimate roughly the spin density remaining on the nitrogen atom of NO from the hyperfine splitting constants according to the method of McNeil et al. (1965). The results are included in Table II. For all of the NO complexes of ferrous cytochrome P-450$_{scc}$, only 20–30% of the spin density remained on the nitrogen atom of NO. The spin density on the nitrogen atom was plotted against another EPR parameter, Δg_{x-y} ($=g_x - g_y$), which was arbitrarily introduced to measure the anisotropy of g values. For various NO complexes of ferrous cytochrome P-450$_{scc}$, there was a good correlation between these two parameters except for the 20(S)-hydroxycholesterol complex as shown in Figure 6. As the anisotropy of g values increases, the spin density on NO decreases. It is apparent from this figure that the NO complex of ferrous cytochrome P-450$_{scc}$ in the presence of 20(S)-hydroxycholesterol cannot be classified as having rhombic symmetry.

It is interesting to note that the minor species at g = 2.03 could be observed for all ferrous–NO complexes except for the 22(R)-hydroxycholesterol complex. Yonetani et al. (1972) pointed out the presence of a similar kind of species, labeled $g_?$ at g = 2.03, in ferrous–NO–myoglobin complex, which corresponds to the species II analyzed by single-crystal EPR measurements for a ferrous–myoglobin–NO complex by Hori et al. (1981). The presence of this kind of species was later reported for the NO complex of ferrous cytochrome P-450$_{LM}$

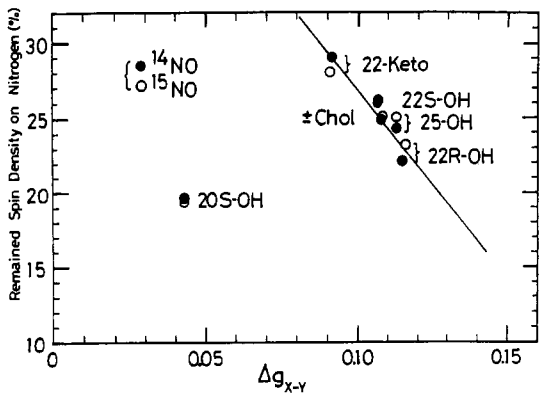

FIGURE 6: Relationship between the anisotropy of the g value ($\Delta g_{x-y} = (g_x - g_y)$) and the spin density remaining on the nitrogen atom of nitric oxide for NO complexes of ferrous cytochrome P-450$_{scc}$ in the presence of various steroids for ^{14}NO complexes (solid circles) and for ^{15}NO complexes (open circles), respectively. The abbreviations used are the same as in Figure 1.

(O'Keefe et al., 1978), although they thought this signal was an indication of the presence of the denatured form. The close proximity of the g value (g = 2.03) to the g_x value of the 20(S)-hydroxycholesterol complex suggests that these two species can be classified as the same type. It was reported that the NO ferrous heme imidazole complex in less polar solvents (CH_3Cl and CH_2Cl_2) gave the EPR spectrum with rhombic symmetry, while in polar solvents (H_2O and dimethyl sulfoxide) less rhombic EPR spectrum could be obtained at 77 K (Kon & Kataoka, 1969). Therefore the EPR spectrum with less rhobmic symmetry may suggest polar surroundings of the ferrous heme NO center of the cytochrome P-450, while the EPR spectrum with rhombic symmetry may indicate less polar surroundings. Later Yoshimura et al. (1979), Morse and Chan (1980), and Hori et al. (1981) investigated the temperature dependence of EPR spectra of NO complexes of ferrous–heme model systems and hemoproteins and demonstrated the existence of the two molecular species in thermal equilibrium.

There may exist a thermal equilibrium between two stable conformations (one with polar and the other with less polar environment around the heme–NO center) with different bonding characteristics of the Fe–N–O moiety at 77 K for the NO complex of ferrous cytochrome P-450$_{scc}$. In the absence of substrate or in the presence of 22(R)-hydroxycholesterol, only one type of Fe–N–O conformation is favored. When cholesterol, 25-hydroxycholesterol, 22(S)-hydroxycholesterol, and 22-ketocholesterol are bound to the substrate-binding site of the enzyme, another potential well for Fe–N–O bonding geometry appears, but still the former type of bonding geometry dominates at 77 K. It must be mentioned that there are many variations within the dominant conformation itself from 22-ketocholesterol to 22(R)-hydroxycholesterol. The minor modification at side-chain groups of these steroids might act as perturbation for the dominant type of Fe–N–O bonding geometry, whereas upon binding of 20(S)-hydroxycholesterol to the substrate-binding site a drastic conformational change must take place. Under this new conformation, the latter type of Fe–N–O geometry is now favored; thus, the environment of the Fe–N–O moiety might become polar.

Correlation between Ferric Low-Spin States and Ferrous–NO Complex States. There was good correlation between ferric low-spin EPR signals and ferrous–NO signals. In the EPR spectra of ferric low-spin species, the 22(R)-hydroxycholesterol complex and the 20(S)-hydroxycholesterol complex showed two extremes of anisotropy (g_z = 2.463 and 2.400, respectively) (Figure 1). In the EPR spectra of the ferrous–NO complex, these two hydroxycholesterol complexes manifested two extremes of anisotropy also, although the direction was reversed. It is interesting to note that other steroid complexes showed the intermediate species between these two extremes in both ferric low-spin and ferrous–NO complex states.

Although we do not know the sixth ligand of the heme in the ferric low-spin state of cytochrome P-450$_{scc}$ now, H_2O or OH^- is most likely the sixth ligand according to the X-ray crystallographic studies of cytochrome P-450$_{cam}$ (Poulous et al., 1985, 1986). If H_2O or OH^- is the sixth ligand, instead of a distal amino acid residue, it is very likely that the similar conformation is adopted by each cytochrome P-450$_{scc}$–hydroxycholesterol complex in both ferric low-spin and ferrous–NO complex states. The present results seem to support this view.

Biological Significance of 20S-Hydroxylation. The perturbations in ferrous–NO EPR signals by a series of steroids and the drastic conformational change upon binding of 20-(S)-hydroxycholesterol as observed by the present study must be reflections of the conformational flexibility in the active site of cytochrome P-450$_{scc}$ and the biological significance of 20S-hydroxylation.

It is very interesting to consider the reason why only binding of 20(S)-hydroxycholesterol to the substrate-binding site of the enzyme caused so significant a conformational change around the heme. Physiologically, 20(S)-hydroxycholesterol is not a proper intermediate of the cholesterol side-chain cleavage reaction (Burstein & Gut, 1976; Larroque et al., 1981; Hume et al., 1984), although the hydroxyl group at the 20S position is stereochemically correct to advance the side-chain cleavage reaction. Indeed, the side-chain cleavage reaction still occurs with 20(S)-hydroxycholesterol as substrate but at a reduced rate (Morisaki et al., 1976). Thus the hydroxylation at the 22R position prior to the hydroxylation at 20S is absolutely required to promote the side-chain cleavage reaction properly (Burstein & Gut, 1976). When the second hydroxylation occurs at the 20S position, newly formed interactions (steric or hydrogen bonding) between the 20(S)-hydroxyl group and the surrounding amino acid residues and porphyrin ring may cause a drastic conformational change of the side-chain group relative to the heme group. Only under this new conformation can the final oxidative cleavage of the C20–C22 bond of the diol take place to yield pregnenolone and isocaproic aldehyde. If the first hydroxylation occurs at the 20S position, the adoptive new conformation (which may or may not be the same with the adoptive conformation for 20(R),22(R)-dihydroxycholesterol mentioned above) is no longer favorable for 22R-hydroxylation, leading to the retarded side-chain cleavage rate.

From our point of view, results obtained from recent resonance Raman experiments on carbon monoxide complexes of ferrous cytochrome P-450$_{scc}$ (Tsubaki & Ichikawa, 1985; Tsubaki et al., 1986a, 1987) are very interesting. These studies suggested that (1) strong steric interactions between heme-bound carbon monoxide and the 22(R)-hydroxyl group or the 22(R)-hydrogen of the side chain exist and (2) the hydroxylation at the 20S position in the first step causes a shift or rotation of the side-chain group relative to the heme. The latter conclusion is consistent with our present results. To prove our hypothesis, the EPR measurements of the NO complex of ferrous cytochrome P-450$_{scc}$ in the presence of 20(R),22(R)-dihydroxycholesterol are highly necessary.

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Registry No. Cholesterol, 57-88-5; steroid 20-22-desmolase, 37292-81-2; 22-ketocholesterol, 19243-30-2; 22(*S*)-hydroxycholesterol, 22348-64-7; 25-hydroxycholesterol, 2140-46-7; 22(*R*)-hydroxycholesterol, 17954-98-2; 20(*S*)-hydroxycholesterol, 516-72-3.

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