Electron Paramagnetic Resonance Study of Ferrous Cytochrome P-450$_{scc}$–Nitric Oxide Complexes: Effects of 20(R),22(R)-Dihydroxycholesterol and Reduced Adrenodoxin†

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ABSTRACT: Electron paramagnetic resonance (EPR) spectra of ferrous–nitric oxide (^{14}NO and ^{15}NO) cytochrome P-450$_{scc}$ complexed with 20(R),22(R)-dihydroxycholesterol were measured at 77 K with X-band (9.35 GHz) microwave frequency. The EPR spectra clearly showed the spin system to have rhombic symmetry (g_x = 2.068, g_z = 2.001, g_y = 1.961, and A_z = 1.89 mT for ^{14}NO) and were distinct from those of 20(S)-hydroxycholesterol complexes. The unique nature of the 20(S)-hydroxycholesterol complexes indicates that 20(S)-hydroxycholesterol is not a proper intermediate in the cholesterol side-chain cleavage reaction. In addition, among various steroid complexes of ferrous–NO species having rhombic symmetry, the EPR spectra of 20(R),22(R)-dihydroxycholesterol complexes were significantly different from those of 22(R)-hydroxycholesterol complexes, suggesting that upon 20S-hydroxylation of 22(R)-hydroxycholesterol the conformation of the active site changes so as to facilitate subsequent cleavage of the C20–C22 bond of the cholesterol side chain. Addition of reduced adrenodoxin to the ferrous–NO cytochrome P-450$_{scc}$ complex in the presence of cholesterol caused a complete shift of the g_x = 2.070 signal to g_x = 2.075, indicating a reorientation of cholesterol in the substrate-binding site of the enzyme upon adrenodoxin binding. Without reduced adrenodoxin, the process of reorientation of cholesterol in the substrate-binding site was very slow, requiring more than 50 h of incubation at 0 °C. The present observations suggest that adrenodoxin may have another positive role in the cholesterol side-chain cleavage reaction, in addition to transferring an electron to the heme of cytochrome P-450$_{scc}$.

The cholesterol side-chain cleavage reaction catalyzed by cytochrome P-450$_{scc}$ located in the inner mitochondrial membrane of the adrenal cortex involves three consecutive hydroxylation steps. The first hydroxylation occurs at the 22R position to yield 22(R)-hydroxycholesterol, the second hydroxylation occurs at the 20S position to give 20(R),22(R)-dihydroxycholesterol, and the third oxidative cleavage of the C20–C22 bond of the diol results in the formation of the pregnenolone and isocaproic aldehyde (Burstein et al., 1975; Burstein & Gut, 1976; Hume et al., 1984). The increased affinity of the hydroxylated intermediates to the enzyme and the increased stability of the ferrous–dioxygen complex in each step (Tuckey & Kamin, 1982, 1983) suggest the existence of strict stereochemistry among substrate, ferrous heme, and heme-bound dioxygen in the cytochrome P-450$_{scc}$ active site which might be situated in a hydrophobic pocket of the polypeptide chain to promote the side-chain cleavage reaction properly (Heyl et al., 1986; Tsubaki et al., 1987b).

Previously, we reported the electron paramagnetic resonance (EPR)[1] spectra of ferrous–nitric oxide derivatives of cytochrome P-450$_{scc}$ at 77 K for the first time (Tsubaki et al., 1987a), which were very similar to those of cytochrome P-450$_{cam}$ (from *Pseudomonas putida*) and cytochrome P-450$_{LM}$ (from rat liver microsomes) (Ebel et al., 1975; O'Keefe et al., 1978). Furthermore, we found that, upon addition of substrate (or its analogues), the EPR spectra exhibited many variations having rhombic symmetry and that only 20(S)-hydroxycholesterol caused an unusual change leading to EPR signals with quasi-axial symmetry. These observations prompted us to speculate that in the cholesterol side-chain cleavage reaction the hydroxylation at the 20S position in the first step will cause a conformational change around the heme, which is no longer favorable for subsequent 22R-hydroxylation, leading to the retarded side-chain cleavage rate (Morisaki et al., 1976; Tsubaki et al., 1987a). Probably this is the reason why the enzyme hydroxylates at the 22R position first (Hume et al., 1984). In the present study, we have extended our EPR study to the ferrous–NO complex of cytochrome P-450$_{scc}$ in the presence of 20(R),22(R)-dihydroxycholesterol to prove our hypothesis.

In addition to the substrate-binding site, the adrenodoxin binding site of cytochrome P-450$_{scc}$ has an essential role in the steroidogenic electron transport for heme reduction and subsequent oxygen activation. However, there is no available information on the interaction between reduced adrenodoxin and ferrous cytochrome P-450$_{scc}$ in the ligated state at present. If reduced adrenodoxin is added to the ferrous–dioxygen de-

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[1] Abbreviations: EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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rivative of cytochrome P-450$_{scc}$ in the presence of substrate, a spontaneous transfer of the second electron can take place, leading to the activation of heme-bound oxygen followed by the hydroxylation of the side-chain group (Hume et al., 1984). Since the ferrous–NO derivatives of cytochrome P-450$_{scc}$ cannot accept the second electron from reduced adrenodoxin, we can expect to observe the interaction between reduced adrenodoxin and ferrous cytochrome P-450$_{scc}$ in ligated state. We have examined the effect of addition of the reduced adrenodoxin to the ferrous–NO derivatives of cytochrome P-450$_{scc}$ in the presence or absence of various substrates by EPR spectroscopy.

Experimental Procedures

Materials. Cholesterol, 22(*R*)-hydroxycholesterol, 22(*S*)-hydroxycholesterol, 20(*S*)-hydroxycholesterol, and 20-ketocholesterol were obtained from Sigma Chemical Co. 25-Hydroxycholesterol was obtained from Steraloids Inc. (Wilton, NH). 20(*R*),22(*R*)-Dihydroxycholesterol was synthesized and purified as previously described (Morisaki et al., 1977). Its authenticity was assured by measurement of the mass spectrum. Nitric oxide (^{14}NO) gas was purchased from Seitetsu Kagaku (Osaka, Japan). Isotopically labeled Na-$^{15}NO_2$ was obtained from Merck and Co. Other chemicals, including glycerol, NaCl, EDTA, KOH, and $Na_2S_2O_4$, were obtained from Wako Pure Chemicals, Inc. (Osaka, Japan), 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). Endogeneous substrates were removed during the purification (Tsubaki et al., 1986b). The sample was then dialyzed against 20 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 100 mM NaCl, and 0.1 mM EDTA. The specific heme content of the sample was more than 15 nmol of P-450/mg of protein. The sample was homogeneous on SDS–polyacrylamide gel electrophoresis and was practically free from the P-420 form. Cytochrome P-450$_{scc}$–substrate (or its analogues) complex was prepared as previously described (Tsubaki et al., 1987a) and was concentrated by centrifugation at 3000 rpm with CENTRIFLO membrane cones (type CF25, Amicon Corp.) to about 0.5–0.6 mM. Adrenodoxin was purified from bovine adrenocortical mitochondria as previously described (Hiwatashi et al., 1986), concentrated to about 1 mM, and dialyzed against 20 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 100 mM NaCl, and 0.1 mM EDTA. Adrenodoxin was added in excess to cytochrome P-450$_{scc}$–substrate complex with a 1:1.2 ratio, and the mixture was incubated at 0 °C overnight to allow equilibration.

Measurements of EPR Spectra. EPR measurements were carried out at 77 or 4.2 K and at X-band (9.35 GHz) microwave frequency with a home-built EPR spectrometer with a 100-kHz field modulation by use of a Varian X-band cavity. Preparation of the ferrous–NO complex of cytochrome P-450$_{scc}$ in the presence or absence of adrenodoxin was done as described previously (Tsubaki et al., 1987a). Adrenodoxin samples in EPR tubes with screw-cap septa were deoxygenated by repeated evacuation and flushing of oxygen-free N_2 gas. Solid sodium dithionite ($Na_2S_2O_4$) was anaerobically introduced to reduce the oxidized form of adrenodoxin. For preparation of the ferrous–carbon monoxide (CO) cytochrome P-450$_{scc}$–reduced adrenodoxin complex, CO gas was anaerobically introduced after the reduction with sodium dithionite. Accuracy of the *g* values is approximately ±0.001, whereas that of hyperfine coupling constants is approximately ±0.05 mT in the present study.

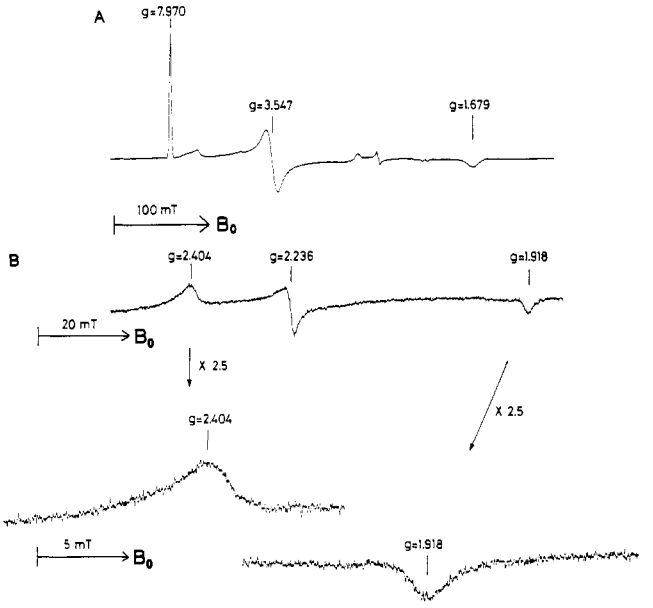

FIGURE 1: EPR spectra of cytochrome P-450$_{scc}$ complexed with 20(*R*),22(*R*)-dihydroxycholesterol for ferric high-spin species (A) and low-spin species (B). Conditions of EPR spectroscopy: microwave frequency, 9.35 GHz (X-band); microwave power, 5 mW; modulation frequency, 100 kHz; amplitude, 0.3 mT; temperature, (A) 4.2 K and (B) 77 K, respectively.

Results

Effect of 20(R),22(R)-Dihydroxycholesterol in the Oxidized State. Cytochrome P-450$_{scc}$ as isolated was almost in pure ferric low-spin form as described previously (Tsubaki et al., 1986a). Upon addition of 20(*R*),22(*R*)-dihydroxycholesterol to substrate-free cytochrome P-450$_{scc}$, a dramatic increase of the high-spin species was observed as shown in the EPR spectrum at 4.2 K (Figure 1A). Nevertheless, 20(*R*),22(*R*)-dihydroxycholesterol produced a new ferric low-spin heme species with $g_z = 2.404$[2] and $g_x = 1.918$ as shown in the EPR spectrum at 77 K (Figure 1B). Orme-Johnson et al. (1979) reported that the EPR spectra of the ferric low-spin species of the 20(*R*),22(*R*)-dihydroxycholesterol complex showed the existence of at least two components, one with $g_z = 2.432$ and $g_x = 1.945$ and the other with $g_z = 2.411$ and $g_x = 1.920$ at 13 K. The latter component is very likely the same species as the one reported in the present study and was clearly distinct from the low-spin species in the substrate-free state ($g_z = 2.417$, $g_x = 1.910$) (Tsubaki et al., 1987a).

The content of the low-spin species was estimated roughly by a comparison of double integrations of the EPR spectra at 77 K for a fully low-spin complex (such as steroid-free cytochrome P-450$_{scc}$) and for the 20(*R*),22(*R*)-dihydroxycholesterol complex. Such an estimate gave the low-spin content to be about 35% of the total heme species of this steroid complex. These observations are essentially consistent with the report of Orme-Johnson et al. (1979). [However, more than 90% of the heme was converted to the high-spin form as judged by light absorption spectrum at 20 °C (data not shown). The reason for this discrepancy is not clear at this stage.]

[2] From the single-crystal EPR analyses for the ferric low-spin derivatives of myoglobin and cytochrome *c* peroxidase, the direction of the maximal *g* value is nearly parallel to that of the heme normal for the high-spin species of these hemoproteins (*z* axis). In the case of the ferric low-spin heme species of cytochrome P-450$_{scc}$, the low-field signal in the powder EPR spectrum is thus assumed to be the *z* absorption. The directions of the *x* and *y* absorptions are mutually perpendicular and might be parallel to the heme plant.

Table I: Effects of Reduced Adrenodoxin on the Electron Paramagnetic Parameters of Nitric Oxide (^{14}NO and ^{15}NO) Complexes of Cytochrome P-450_{scc}

complex		−adrenodoxin		+adrenodoxin	
		g values	hyperfine coupling constants (mT)	g values	hyperfine coupling constants (mT)
substrate-free (−Chol)	^{14}NO	$g_x = 2.071$	$A_x = 1.00$	$g_x = 2.071$	$A_x = 0.97$
		$g_z = 2.001$	$A_z = 2.20$	$g_z = 2.002$	$A_z = 2.16$
		$g_y = 1.962$	$A_y = 1.10$	$g_y = 1.960$	$A_y = 1.12$
				($g_x = 2.102$	$A_x = 1.20$)[a]
	^{15}NO	$g_x = 2.070$	$A_x = 1.40$	$g_x = 2.070$	$A_x = 1.32$
		$g_z = 2.000$	$A_z = 3.05$	$g_z = 1.999$	$A_z = 3.05$
		$g_y = 1.962$	$A_y = 1.50$	$g_y = 1.961$	$A_y = 1.46$
				($g_x = 2.102$	$A_x = 2.02$)[a]
cholesterol (+Chol)	^{14}NO	$g_x = 2.071$	$A_x = 1.00$	$g_x = 2.075$	$A_x = 0.95$
		$g_z = 2.001$	$A_x = 2.20$	$g_z = 2.001$	$A_z = 2.09$
		$g_y = 1.962$	$A_y = 1.10$ (25.2%)[b]	$g_y = 1.961$	$A_y = 1.05$ (23.8%)[b]
	^{15}NO	$g_x = 2.070$	$A_x = 1.40$	$g_x = 2.075$	$A_x = 1.39$
		$g_z = 2.000$	$A_z = 3.05$	$g_z = 2.000$	$A_z = 2.87$
		$g_y = 1.962$	$A_y = 1.50$ (24.9%)[b]	$g_y = 1.961$	$A_y = 1.43$ (22.7%)[b]

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

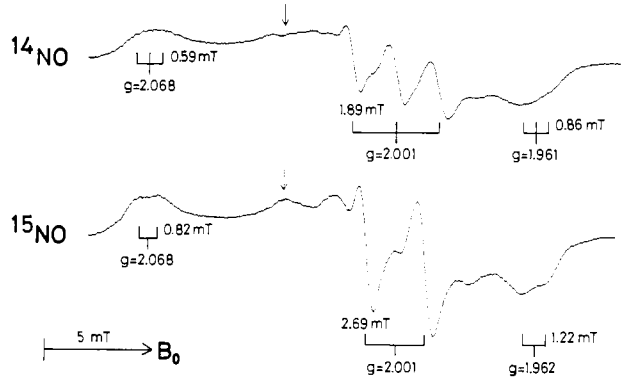

FIGURE 2: EPR spectra of ferrous-^{14}NO (upper) and -^{15}NO (lower) complexes of cytochrome P-450_{scc} in the presence of 20(*R*),22(*R*)-dihydroxycholesterol. Arrows indicate $g_x = 2.03$ signals. The conditions for EPR spectroscopy were the same as in Figure 1B.

Nitric Oxide Complex of Ferrous Cytochrome P-450_{scc} in the Presence of 20(R),22(R)-Dihydroxycholesterol. Both ^{14}NO and ^{15}NO complexes of ferrous cytochrome P-450_{scc} in the presence of 20(*R*),22(*R*)-dihydroxycholesterol exhibited EPR signals centered around $g = 2$ having rhombic symmetry ($g_x = 2.068$, $g_z = 2.001$, and $g_y = 1.961$) at 77 K (Figure 2). The change from a well-resolved triplet to a doublet with a concomitant increase in the hyperfine splitting at $g_z = 2.001$ from 1.89 to 2.69 mT upon the replacement of ^{14}NO with ^{15}NO was consistent with the nuclear spin (I) and the gyromagnetic ratio (γ_N) of ^{14}N [$I = 1$ and $\gamma_N = 1934$ rad/(s·G)] and ^{15}N [$I = {}^1/_2$ and $\gamma_N = -2712$ rad/(s·G)] nuclei. Thus, these hyperfine splittings in the central resonance were derived from the hyperfine interaction of the unpaired electron with the nitrogen nucleus of the bound NO groups. The line shapes of the two signals at low and high fields were also affected by the isotopic substitution. For the ^{15}NO complex, the signals at $g = 2.068$ and $g = 1.961$ exhibited well-resolved hyperfine splittings of 0.82 and 1.22 mT, respectively. However, direct determination of the hyperfine splittings in corresponding signals for the ^{14}NO complex was not possible because of poor spectral resolution. Therefore, we estimated these hyperfine splittings from the assumption of $A(^{14}NO) = A(^{15}NO)[A_z(^{14}NO)/A_z(^{15}NO)]$.

The minor species at $g = 2.03$ was also observed for these complexes as shown in Figure 2 (indicated by arrows). We speculated previously that the EPR signals with less rhombic (or quasi-axial) symmetry are characteristic of the polar surroundings of the ferrous–NO heme moiety of the enzyme (Tsubaki et al., 1987a). In the present study there was no formation of the P-420 form even after overnight incubation at 0 °C when 20(*R*),22(*R*)-dihydroxycholesterol was included in the ferrous–NO complex of cytochrome P-450_{scc}.

Effect of Ferrous–CO Cytochrome P-450_{scc} on the EPR Spectrum of Reduced Adrenodoxin. To see the effect of the association of reduced adrenodoxin with cytochrome P-450_{scc} in the ferrous-ligated state on adrenodoxin itself, we compared the EPR spectrum of reduced adrenodoxin alone with that of reduced adrenodoxin complexed with ferrous–CO cytochrome P-450_{scc} in the absence of substrate. There was no difference in the EPR spectra between these two species; g values [$g = 2.020$ and $g = 1.933$, consistent with the data previously reported (Orme-Johnson and Sands, 1973)] and spectral shapes remained the same (spectra not shown). Inclusion of various substrates [cholesterol, 22(*R*)-hydroxycholesterol, 20(*S*)-hydroxycholesterol, and 20(*R*),22(*R*)-dihydroxycholesterol] in the ferrous–CO cytochrome P-450_{scc} complexed with the reduced adrenodoxin did not cause any change at all.

Effect of Reduced Adrenodoxin on the EPR Spectrum of the Ferrous–NO Complex of Cytochrome P-450_{scc}. Addition of reduced adrenodoxin to the ferrous–NO complex of cytochrome P-450_{scc} in the absence of substrate caused no change in the EPR spectrum compared to that of the original ferrous–NO complex except for the overlapped EPR signals from the reduced adrenodoxin (Figure 3). The EPR signals from the reduced adrenodoxin itself did not show any change of g values nor spectral line shape, as seen for the ferrous–CO cytochrome P-450_{scc} complexed with the reduced adrenodoxin. In addition, a minor new species could be seen in the EPR spectrum. This species was characterized by the appearance of a $g_x = 2.102$ signal having well-resolved triplet and doublet splittings for ^{14}NO and ^{15}NO complexes, respectively (Table I); but we did not pursue further the nature of this minor species in the present study. We also examined the effects of addition of reduced adrenodoxin to ferrous–NO complexes of cytochrome P-450_{scc} in the presence of 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, 22(*S*)-hydroxycholesterol, 25-hydroxycholesterol, 22-ketocholesterol, and 20(*R*),22(*R*)-dihydroxycholesterol, respectively. In all cases there was no change in the ferrous–NO signals nor in the reduced adrenodoxin signals (spectra not shown).

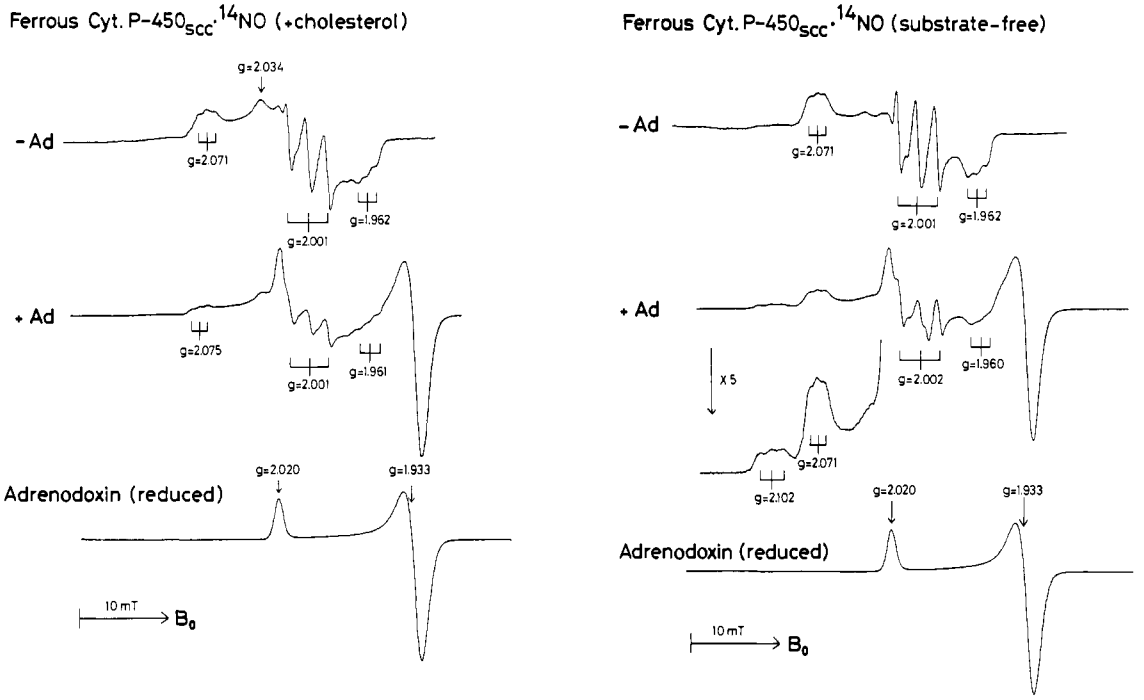

FIGURE 3: Effect of reduced adrenodoxin on the EPR spectra of the ferrous–NO complex of cytochrome P-450$_{scc}$ in the absence (right) and in the presence (left) of cholesterol. Top (–Ad), without reduced adrenodoxin; middle (+Ad), in the presence of reduced adrenodoxin; bottom (Ad), reduced adrenodoxin only. The conditions for EPR spectroscopy were the same as in Figure 1B.

However, addition of reduced adrenodoxin to the ferrous–NO complex of cytochrome P-450$_{scc}$ in the presence of cholesterol caused a complete shift of the g_x = 2.071 signal to g_x = 2.075 (Figure 3 and Table I). [The EPR signals at g_z = 2.001 and g_y = 1.961, however, seemed to change only slightly (A_z values decreased slightly)]. This new g_x = 2.075 signal gave splittings into a triplet (A_x = 0.95 mT) and a doublet (A_x = 1.39 mT) for ^{14}NO and ^{15}NO complexes, respectively (Table I). The EPR signals from the reduced adrenodoxin in this complexed state, however, did not show any changes compared to those in the free state.

Time-Dependent Change of the EPR Spectrum of the Ferrous–NO Complex of Cytochrome P-450$_{scc}$ in the Presence of Cholesterol. We noticed that the EPR spectrum of the ferrous–NO complex of cytochrome P-450$_{scc}$ in the presence of cholesterol (but in the absence of adrenodoxin) showed a very slow but distinct time-dependent change during the incubation at 0 °C. To clarify the time dependency of this change, we designed a series of EPR measurements for the ferrous–NO complex of cytochrome P-450$_{scc}$ in the presence of cholesterol. The ferrous–NO (^{14}NO or ^{15}NO) complexes were prepared under anaerobic condition and were incubated at 0 °C and then frozen by immersion into liquid nitrogen at appropriate intervals. During the first several hours of the incubation at 0 °C, there was only one kind of EPR species characterized by the g_x = 2.070 signal (we call this species the g_x = 2.070 species hereafter), which had very similar g values with that of the ferrous–NO complex without cholesterol (Tsubaki et al., 1987a) (Figure 4 and Tables I and II). But prolonged incubation at 0 °C led to the appearance of a new EPR species overlapping the g_x = 2.070 signal. After about 50 h of incubation at 0 °C, the g_x = 2.070 species disappeared almost completely, and a new EPR species with a g_x = 2.075 signal (we call this new EPR species the g_x = 2.075 species hereafter) dominated in the spectrum. Some of the samples were thawed after the EPR measurements and were incubated at 0 °C for a further appropriate period of time and then refrozen, and their EPR spectra were remeasured.

Table II: Time-Dependent Change of EPR Parameters of the Ferrous–NO Complex of Cytochrome P-450$_{scc}$ in the Presence of Cholesterol

	initial species (g_x = 2.070 species)		final species (g_x = 2.075 species)	
complex	g values	hyperfine coupling constants (mT)	g values	hyperfine coupling constants (mT)
^{14}NO	g_x = 2.070	A_x = 0.97	g_x = 2.075	A_x = 0.95
	g_z = 2.000	A_z = 2.19	g_z = 2.000	A_z = 2.09
	g_y = 1.961	A_y = 1.10 (24.8%)[a]	g_y = 1.961	A_y = 1.06 (23.7%)[a]
^{15}NO	g_x = 2.070	A_x = 1.32	g_x = 2.075	A_x = 1.29
	g_z = 2.000	A_z = 3.01	g_z = 2.000	A_z = 2.85
	g_y = 1.962	A_y = 1.50 (24.8%)[a]	g_y = 1.962	A_y = 1.57 (22.2%)[a]

[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). The estimated spin densities for the g_x = 2.070 species are in good agreement with those of the substrate-free form as previously reported (Tsubaki et al., 1987a) and with those of the cholesterol complex without adrenodoxin (Table I).

This thawing and the refreezing cycling did not cause any significant effect on the EPR spectrum. There was no formation of the P-420 form at least during the incubation at 0 °C (up to 96 h). Addition of reduced adrenodoxin afterward to the g_x = 2.075 species under anaerobic conditions did not cause any further change in the EPR spectrum except for the overlapping of the EPR signals originating from the reduced adrenodoxin itself.

Discussion

Effect of 20(R),22(R)-Dihydroxycholesterol. The addition of cholesterol, 25-hydroxycholesterol, or 20(R),22(R)-dihydroxycholesterol to ferric cytochrome P-450$_{scc}$ in the substrate-free form causes a significant decrease in the low spin heme concentration. Among these steroids, 20(R),22(R)-dihydroxycholesterol is the strongest high-spin inducer (Orme-Johnson et al., 1979). The mechanism of the induction

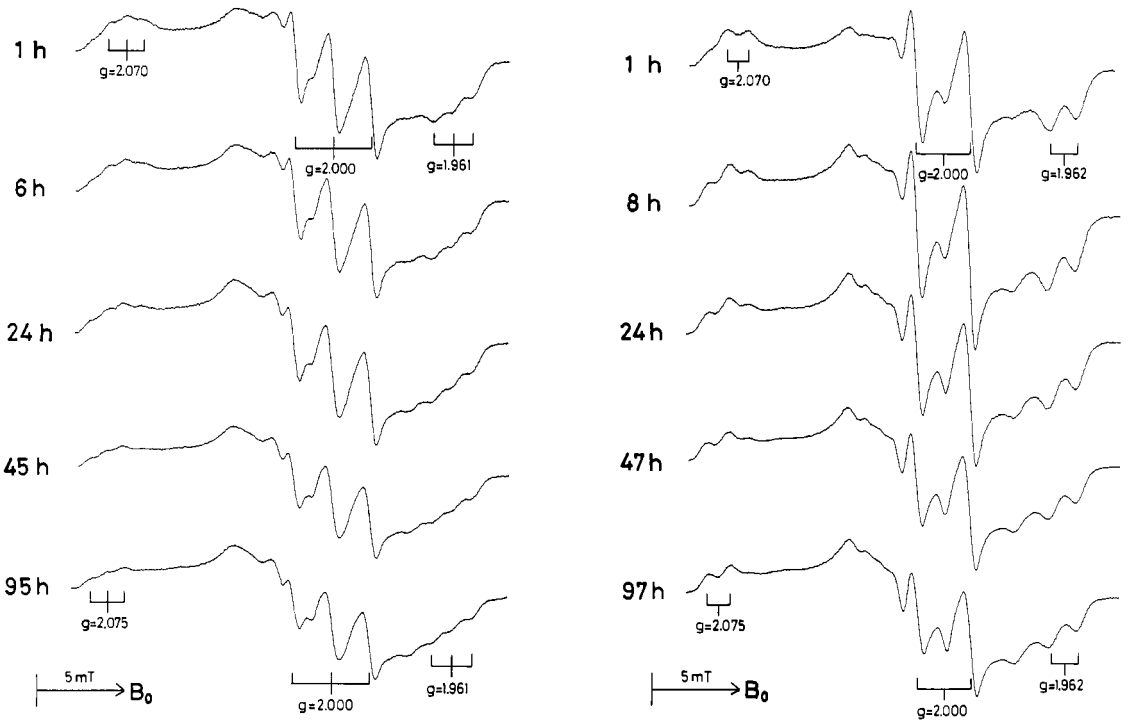

FIGURE 4: Time-dependent change of EPR spectra of ferrous–^{14}NO (left) and –^{15}NO (right) complexes of cytochrome P-450$_{scc}$ in the presence of cholesterol. Samples were incubated on ice for the indicated periods after the ferrous–NO complex was prepared anaerobically, and then, their EPR spectra were measured at 77 K. The conditions for EPR spectroscopy were the same as in Figure 1B.

of the high-spin species upon substrate binding is explained as the access of the sixth ligand (oxygen atom of water molecule) to the heme iron being inhibited by the steric hindrance of the substrate side-chain group leading to the pentacoordinated high-spin species [Coon & White, 1980; Jefcoate, 1986; Murray et al. (1985) and references cited therein]. This hypothesis is supported by recent X-ray crystallographic data for cytochrome P-450$_{cam}$ in the presence or absence of substrate (Poulos et al., 1985, 1986, 1987); in the absence of substrate (camphor), a cluster of water molecules occupies the sixth ligating position, while the cytochrome P-450$_{cam}$–camphor complex adopted a pentacoordinated ferric high-spin heme. 20(*R*),22(*R*)-Dihydroxycholesterol has two hydroxyl groups with the same stereochemical configurations as those of 20(*S*)-hydroxycholesterol and 22(*R*)-hydroxycholesterol, respectively; those sterols were, however, strong low-spin inducers. This fact indicates that a drastic conformational change must take place among the side-chain group of substrate, the heme, and the surrounding amino acid residues when the second hydroxylation occurs at the 20*S* position of the side-chain group of 22(*R*)-hydroxycholesterol [or at the 22*R* position of 20(*S*)-hydroxycholesterol].

Despite the strong high-spin inducibility of 20(*R*),22(*R*)-dihydroxycholesterol, we could observe the ferric low-spin species of cytochrome P-450$_{scc}$ in the presence of this steroid in the EPR spectrum at 77 K (Orme-Johnson et al., 1979). The side-chain group of 20(*R*),22(*R*)-dihydroxycholesterol in the substrate binding site inhibits the access of a water molecule to the heme iron very strongly, but not completely; thus, a partial occupation of the sixth ligating position by a water molecule might still occur, but the immediate environment of this ferric low-spin heme is distinct from that of the substrate-free form. Indeed, the EPR parameters indicate that this low-spin species (g_z = 2.404 and g_x = 1.918) is different from the ferric low-spin species of cytochrome P-450$_{scc}$ in the absence of substrate (g_z = 2.417 and g_x = 1.910) (Tsubaki et al., 1987a).

Analyses of our present EPR data for ferrous–NO species of cytochrome P-450$_{scc}$ complexed with 20(*R*),22(*R*)-dihydroxycholesterol by the same method as previously described (Tsubaki et al., 1987a) showed that this EPR species could be classified as the usual ferrous–NO species having rhombic symmetry (Δg_{x-y} = 0.106 and total spin density = 25.2% for ^{15}NO complex) and was distinct from those of 20(*S*)-hydroxycholesterol complex (Tsubaki et al., 1987a). The unique nature of the 20(*S*)-hydroxycholesterol complex among various steroid complexes examined by EPR spectroscopy indicates that 20(*S*)-hydroxycholesterol is not a proper intermediate in the cholesterol side-chain cleavage reaction (Larroque et al., 1981; Hume et al., 1984). Probably, upon binding of 20(*S*)-hydroxycholesterol, a drastic conformational change might occur at the active site (the substrate-binding site, the heme, and their immediate vicinity), leading to an unfavorable environment (polar or hydrophilic) for the subsequent 22(*R*)-hydroxylation reaction (Morisaki et al., 1976) or to an undesirable conformation for the stability of the enzyme itself.

Furthermore, among the various steroid complexes of ferrous–NO species having rhombic symmetry, the EPR parameters of the 20(*R*),22(*R*)-dihydroxycholesterol complex were very similar to those of the 22(*S*)-hydroxycholesterol complex rather than to those of the 22(*R*)-hydroxycholesterol complex (Tsubaki et al., 1987a). This fact suggests that upon 20*S*-hydroxylation of 22(*R*)-hydroxycholesterol the conformation of the active site changes so as to facilitate the subsequent cleavage of the C20–C22 bond and that under this new conformation the environment around the heme might be very similar to those of complexes with 22(*S*)-hydroxycholesterol.

Effect of Cytochrome P-450$_{scc}$ on Reduced Adrenodoxin. It is known that the redox state of adrenodoxin does not appear to have a major effect on its association with cytochrome P-450$_{scc}$; however, the redox state of the cytochrome exerts a larger effect on the interaction with (reduced) adrenodoxin (Lambeth & Pember, 1983). From the viewpoint of a physiological mechanism of steroidogenic electron transport, the interaction between reduced adrenodoxin and ferrous cy-

tochrome P-450$_{scc}$ in ligated states (as a model for an oxygenated intermediate complex) is expected to be stronger than, or, at least, comparable to, that between oxidized adrenodoxin and ferric cytochrome P-450$_{scc}$. Therefore, it is very interesting to examine the effect of the binding of reduced adrenodoxin on the ferrous heme moiety of cytochrome P-450$_{scc}$.

Adrenodoxin can accommodate only a single electron in a 2Fe2S cluster in the reduced state, becoming an EPR-visible species (Estabrook et al., 1973). In the free state, reduced adrenodoxin produced a characteristic EPR spectrum as shown in Figure 3. Binding of the reduced adrenodoxin to the ferrous–CO complex of cytochrome P-450$_{scc}$ in the absence or presence of various substrates did not show any influences in the EPR spectra, suggesting that the active center (2Fe2S cluster) of reduced adrenodoxin has a rigid structure and did not suffered a drastic conformational change upon association with its electron acceptor. This insensitivity of the EPR signals of reduced adrenodoxin was also observed when the reduced adrenodoxin became associated with the ferrous–NO complex of cytochrome P-450$_{scc}$. It is known that when two paramagnetic species come within about 10 Å of each other, dipolar interactions are large enough to affect the shapes of the EPR signals, causing a broadening of the signals (Orme-Johnson & Sands, 1973). But we did not experience such a broadening of EPR signals nor a shift of g values. Therefore, it may be concluded that the distance between the 2Fe2S cluster of reduced adrenodoxin and the ferrous–NO heme of cytochrome P-450$_{scc}$ in the associated form is longer than 10 Å.

Effect of Reduced Adrenodoxin on the Ferrous–NO Complex of Cytochrome P-450$_{scc}$ in the Presence of Cholesterol. There existed clear indications of the influence of reduced adrenodoxin to the heme moiety of cytochrome P-450$_{scc}$ in the EPR spectra when reduced adrenodoxin was associated with the ferrous–NO complex of cytochrome P-450$_{scc}$ in the presence of cholesterol. Upon binding of the reduced adrenodoxin in the presence of cholesterol, the EPR signal at g_x = 2.070 (A_x = 1.00 mT for the ^{14}NO complex) showed a spontaneous shift to g_x = 2.075 (A_x = 0.95 mT for the ^{14}NO complex) (Figure 3). The ferrous–NO complex of cytochrome P-450$_{scc}$ in the presence of cholesterol, but without reduced adrenodoxin, changed gradually its EPR spectrum from the g_x = 2.070 species to the g_x = 2.075 species during incubation at 0 °C (Figure 4 and Table II). Therefore, it may be concluded that the g_x = 2.075 species formed during the incubation at 0 °C without reduced adrenodoxin was identical with the new EPR species formed upon binding of reduced adrenodoxin in the presence of cholesterol. Indeed, analyses of present EPR spectra for these cholesterol complexes with the same method as previously described (Tsubaki et al., 1987) revealed that the EPR parameters for the g_x = 2.075 species coincide with those of EPR species formed upon binding of reduced adrenodoxin in the presence of cholesterol.

We interpret our present observations as follows: In the first several hours of incubation at 0 °C, cholesterol bound to cytochrome P-450$_{scc}$ does not orient properly in the substrate-binding site of cytochrome P-450$_{scc}$ (thus, the g_x = 2.070 species), and it takes a very long period of the incubation at 0 °C to reorient cholesterol appropriately in the substrate-binding site (leading to the g_x = 2.075 species). Addition of reduced adrenodoxin to the incubation mixture accelerates significantly this reorientation process from the g_x = 2.070 to the g_x = 2.075 species. This new species (the g_x = 2.075 species) can be classified as the usual ferrous–NO species with rhombic symmetry, as expected, and the immediate environment of the heme moiety might be very similar to that of the 22(R)-hydroxycholesterol complex (Tsubaki et al., 1987a).

It is very peculiar that only cholesterol can take such a long time to locate itself in a proper substrate-binding site of the cytochrome. Other steroids so far examined seem to orient themselves in a proper site spontaneously, as judged by their EPR spectra. As one possible explanation, the hydrophobicity of cholesterol may be so high that cholesterol cannot locate itself in a proper orientation in a hydrophobic pocket of the substrate-binding site without aid from adrenodoxin, whereas other steroids used are not so hydrophobic as cholesterol, leading to a spontaneous self-orientation in the substrate-binding site without help of adrenodoxin binding. It is very interesting to note that the g_x = 2.075 species has a very similar EPR character to that of ferrous–NO complex of cytochrome P-450$_{scc}$ in the presence of 25-hydroxycholesterol (g_x = 2.074), which is expected to have the same configuration as the cholesterol complex around C20–C22 of the side-chain group relative to the heme in the substrate-binding site of the enzyme. Due to the 25-hydroxyl group in the side chain, however, this hydroxycholesterol is much hydrophilic than cholesterol, leading to the spontaneous self-orientation in the substrate-binding site. Probably this is the reason why 25-hydroxycholesterol is superior by at least twice to cholesterol as the substrate for the side-chain cleavage reaction catalyzed by cytochrome P-450$_{scc}$ (Morisaki et al., 1980).

In conclusion, the active site of cytochrome P-450$_{scc}$ enzyme has the conformational flexibility to process the side-chain cleavage reaction properly and efficiently. It is obvious from the present and the previous studies that during the side-chain cleavage reaction the conformation of the active site changes at least 3 times: first, the reorientation of cholesterol in the substrate-binding site occurs, and this leads to the regiospecific hydroxylation at the 22R position of the side-chain group (however, this process is very slow without reduced adrenodoxin, and therefore, the acceleration of this process by the adrenodoxin binding is essential); the second slight conformational adjustment occurs after the 22R-hydroxylation reaction to lead to the regioselective 20S-hydroxylation; when 20(R),22(R)-dihydroxycholesterol is formed, the third conformational change takes place subsequently to cleave the C20–C22 bond. The second and third conformational changes seem to be independent of the adrenodoxin binding, since there was no effect in the EPR spectra when reduced adrenodoxin was added.

Although our present proposal is based on the ferrous–NO complexes, an almost identical molecular mechanism might be operative in the side-chain cleavage reaction catalyzed by cytochrome P-450$_{scc}$. To clarify the roles of (reduced or oxidized) adrenodoxin in the side-chain cleavage reaction, further investigations are necessary.

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Dynamic Fluorescence Properties of Bacterial Luciferase Intermediates†

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ABSTRACT: Three fluorescent species produced by the reaction of bacterial luciferase from *Vibrio harveyi* with its substrates have the same dynamic fluorescence properties, namely, a dominant fluorescence decay of lifetime of 10 ns and a rotational correlation time of 100 ns at 2 °C. These three species are the metastable intermediate formed with the two substrates $FMNH_2$ and O_2, both in its low-fluorescence form and in its high-fluorescence form following light irradiation, and the fluorescent transient formed on including the final substrate tetradecanal. For native luciferase, the rotational correlation time is 62 or 74 ns (2 °C) derived from the decay of the anisotropy of the intrinsic fluorescence at 340 nm or the fluorescence of bound 8-anilino-1-naphthalenesulfonic acid (470 nm), respectively. The steady-state anisotropy of the fluorescent intermediates is 0.34, and the fundamental anisotropy from a Perrin plot is 0.385. The high-fluorescence intermediate has a fluorescence maximum at 500 nm, and its emission spectrum is distinct from the bioluminescence spectrum. The fluorescence quantum yield is 0.3 but decreases on dilution with a quadratic dependence on protein concentration. This, and the large value of the rotational correlation time, would be explained by protein complex formation in the fluorescent intermediate states, but no increase in protein molecular weight is observed by gel filtration or ultracentrifugation. The results instead favor a proposal that, in these intermediate states, the luciferase undergoes a conformational change in which its axial ratio increases by 50%.

Bacterial luciferase is an enzyme (M_r 77 000; α,β) that reacts with $FMNH_2$, O_2, and a long-chain aliphatic aldehyde to produce bioluminescence (Lee, 1985). The emission spectrum is broad and unstructured, with a maximum in the range of 487-505 nm depending on the type of bacterium from which the luciferase was isolated. Hastings and Gibson (1963) showed that bioluminescence was also produced if the addition of aldehyde was delayed over the other reactants. They proposed that the luciferase-bound $FMNH_2$, which they called intermediate I, reacted with oxygen to form an oxygenated complex, intermediate II.[1] They suggested that the bioluminescence was by a reaction of the aldehyde with II. At room temperature, the bioluminescence potential of II lasted less

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[1] Abbreviations: BSA, bovine serum albumin; ANS, 8-anilino-1-naphthalenesulfonic acid; II, luciferase intermediate II; FT, luciferase fluorescent transient; Q_f, quantum yield of fluorescence; FWHM, full width at half-maximum; DW, Durbin-Watson parameter; τ, fluorescence decay time; ϕ, rotational correlation time; HPLC, high-performance liquid chromatography.

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