Active Site of Bovine Adrenocortical Cytochrome P-450$_{11\beta}$ Studied by Resonance Raman and Electron Paramagnetic Resonance Spectroscopies: Distinction from Cytochrome P-450$_{scc}$[†]

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ABSTRACT: Cytochrome P-450$_{11\beta}$ was purified as the 11-deoxycorticosterone-bound form from bovine adrenocortical mitochondria and its active site was investigated by resonance Raman and EPR spectroscopies. Resonance Raman spectra of the purified sample revealed that the heme iron adopts the pure pentacoordinated ferric high-spin state on the basis of the ν_{10} (1629cm^{-1}) and ν_3 (1490 cm^{-1}) mode frequencies, which are higher than those of the hexacoordinated ferric high-spin cytochrome P-450$_{scc}$–substrate complexes. In the ferrous–CO state, a Fe^{2+}–CO stretching mode was identified at 481.5 cm^{-1} on the basis of an isotopic substitution technique; this frequency is very close to that of cytochrome P-450$_{scc}$ in the cholesterol-complexed state (483 cm^{-1}). The EPR spectra of the purified sample at 4.2 K showed ferric high-spin signals (at g = 7.98, 3.65, and 1.71) that were clearly distinct from the cytochrome P-450$_{scc}$ ferric high-spin signals (g = 8.06, 3.55, and 1.68) and confirmed previous assignments of ferric high-spin signals in adrenocortical mitochondria. The EPR spectra of the nitric oxide (NO) complex of ferrous cytochrome P-450$_{11\beta}$ showed EPR signals with rhombic symmetry (g_x = 2.068, g_z = 2.001, and g_y = 1.961) very similar to those of the ferrous cytochrome P-450$_{scc}$–NO complex in the presence of 22(*S*)-hydroxycholesterol and 20(*R*),22-(*R*)-dihydroxycholesterol at 77 K. These spectral data indicate that the stereochemical structure surrounding the active site of cytochrome P-450$_{11\beta}$ bears a close resemblance to that of cytochrome P-450$_{scc}$ in the ferrous ligated state (ferrous–CO and ferrous–NO states) but not in the ferric high-spin state.

Adrenal cortex mitochondria contain specific cytochrome P-450s responsible for the major steroidogenesis steps such as cholesterol side-chain cleavage (by cytochrome P-450$_{scc}$) and 11β-hydroxylation (by cytochrome P-450$_{11\beta}$). The genes of these two mitochondrial cytochrome P-450s are thought to have evolved from a common ancestor gene (Morohashi et al., 1987). Both cytochrome P-450$_{scc}$ and cytochrome P-450$_{11\beta}$ are synthesized as larger precursor molecules by cytoplasmic ribosomes and are posttranslationally transported to the inner mitochondrial membrane to form mature forms (Kramer et al., 1982; Nabi et al., 1983). Both cytochromes accept electrons required for the oxygen activation from a common iron–sulfur protein, adrenodoxin (Lambeth et al., 1982).

Purified cytochrome P-450$_{11\beta}$ was known to catalyze both 11β- and 18-hydroxylations of 11-deoxycorticosterone (Watanuki et al., 1977, 1978; Sato et al., 1978) and both 11β- and 19-hydroxylations of 18-hydroxy-11-deoxycorticosterone (Momoi et al., 1983, 1985), both in the reconstituted enzymatic systems. Recent researches have revealed that the conversion of 18-hydroxycorticosterone to aldosterone is also catalyzed by the same cytochrome, confirming that a single enzyme species, cytochrome P-450$_{11\beta}$, is responsible for the biogenesis of aldosterone from 11-deoxycorticosterone (Wada et al., 1984, 1985; Yanagibashi et al., 1986, 1988). On the other hand, Katagiri and co-workers have found that cytochrome P-450$_{11\beta}$ also acts as an "aromatase", catalyzing the conversion of 19-hydroxyandrostenedione to 19-oxo-androstenedione and to estrone (Suhara et al., 1986a,b, 1988). Thus the multistep reaction mechanism of cytochrome P-450$_{11\beta}$ shows a remarkable similarity to that of cytochrome P-450$_{scc}$, although the regiospecificity of cytochrome P-450$_{11\beta}$ toward the substrates seems to be much lower than that of cytochrome P-450$_{scc}$ (Ikushiro et al., 1989). The multistep reaction requires a precise readjustment of the spatial relationship between the substrate and the active center at each of the monooxygenase reaction steps. Such a sensitive mechanism has been revealed somewhat in detail for the cytochrome P-450$_{scc}$ system (Orme-Johnson et al., 1979; Tsubaki et al., 1986, 1987a,b, 1988).

Recent evidences suggest the presence of the multiple forms of cytochrome P-450$_{11\beta}$ in mammalian adrenocortices (Lauber et al., 1987; Ohnishi et al., 1988; Ogishima et al., 1989a,b). The deduced amino acid sequences differ by only three amino acid residues in the NH_2-terminal region of the enzyme in bovine adrenal cortices. Whether such an existence of the multiple forms of cytochrome P-450$_{11\beta}$ may be responsible for the regulation of the biosynthesis of the most potent mineralcorticoid, aldosterone, from 11-deoxycorticosterone, remains to be elucidated (Hashimoto et al., 1989; Globerman et al., 1988; Ogishima et al., 1989a,b; Ikushiro et al., 1989). Therefore it is very important to clarify the molecular nature of cytochrome P-450$_{11\beta}$ more precisely to understand the en-

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zymatic mechanism throughout.

However, very little is known about the physicochemical properties of the purified cytochrome P-450$_{11\beta}$, probably due to the instability of the enzyme during the purification steps. In the present study, we have developed a new purification procedure to obtain a large quantity of the purified cytochrome P-450$_{11\beta}$, enabling us to measure the resonance Raman and EPR spectra for the first time.

Experimental Procedures

Purification of Cytochrome P-450$_{11\beta}$. This enzyme was purified from bovine adrenal cortex by a modification of the published method of Suhara et al. (1978) for the first half of the procedure. Adrenocortical mitochondria depleted of adrenodoxin and NADPH-adrenodoxin reductase with sonication were homogenized with 50 mM potassium phosphate buffer (pH 7.4) containing 20 μM deoxycorticosterone, 0.1 mM dithiothreitol, and 0.1 mM EDTA (hereafter referred to as "standard buffer"). The protein concentration was adjusted to 20 mg/mL. The homogenate was treated with 1.2% (w/v) sodium cholate for 1 h at 4 °C with stirring to solubilize cytochrome P-450$_{11\beta}$ and was ultracentrifuged. The supernatant was fractioned by ammonium sulfate precipitation with 0–33% saturated. The precipitate was suspended in the standard buffer and was dialyzed against the standard buffer overnight. After the dialysis, the pellet was collected by centrifugation and was resuspended in the standard buffer. The suspension was then treated with 1.0% (w/v) sodium cholate for 1 h at 4 °C with stirring followed by ultracentrifugation at 100000*g* for 1 h and the supernatant was saved. The concentration of sodium cholate in the supernatant was adjusted to 0.7% (w/v) with the standard buffer. The supernatant was then loaded onto a column of ω-aminooctyl-Sepharose 4B previously equilibrated with the standard buffer containing 0.7% (w/v) sodium cholate. The column was washed with the same buffer extensively. The absorbed cytochrome P-450$_{11\beta}$ was eluted with the standard buffer containing 0.3 M KCl and 1.0% (w/v) sodium cholate. The crude cytochrome P-450$_{11\beta}$ fraction obtained was dialyzed against the standard buffer at 4 °C overnight. During the dialysis the cytochrome P-450$_{11\beta}$ solution became turbid. Precipitated cytochrome P-450$_{11\beta}$ was collected by centrifugation and the resultant pellet was washed with the standard buffer several times at 4 °C. Analysis of the sample at this purification stage by SDS–polyacrylamide gel electrophoresis revealed a negligible contamination from cytochrome P-450$_{scc}$ or other proteins.

The cytochrome P-450$_{11\beta}$ pellet was solubilized with the 5-fold-diluted standard buffer containing 0.5% (v/v) Tween 20 and 1.0% (w/v) sodium cholate. The solubilized cytochrome P-450$_{11\beta}$ was further diluted 5-fold with the standard buffer containing 0.5% (v/v) Tween 20 and the solution was directly applied to an adrenodoxin–Sepharose 4B column (Sugiyama et al., 1976; Chashchin et al., 1984; Tsubaki et al., 1986b) previously equilibrated with the 5-fold-diluted standard buffer containing 0.5% Tween 20 and 0.2% (w/v) sodium cholate. Cytochrome P-450$_{11\beta}$ was adsorbed on the column as a brownish band and the column was washed with the same buffer. Then cytochrome P-450$_{11\beta}$ was eluted with the standard buffer containing 0.5% Tween 20 and 0.2% (w/v) sodium cholate. The cytochrome was concentrated by centrifugation in CENTRIFLO membrane cones (type CF 25, Amicon Corp.). Inclusion of glycerol (20% (v)), a usual ingredient in the buffer during the purification of the membraneous cytochrome P-450, however, was destructive to cytochrome P-450$_{11\beta}$ and, therefore, was not used. Our new preparation method gave a better yield than the previous ones (Suhara et al., 1978; Momoi et al., 1983), probably due to the milder conditions during the purification steps.

Purification of Cytochrome P-450$_{scc}$ and Adrenodoxin. Cytochrome P-450$_{scc}$ was purified from bovine adrenocortical mitochondria as previously described (Tsubaki et al., 1986a). The purified cytochrome P-450$_{scc}$ was complexed with various hydroxycholesterols as previously described (Tsubaki et al., 1987a). Adrenodoxin was purified from bovine adrenocortical mitochondria as previously described (Hiwatashi et al., 1986). Adrenodoxin–Sepharose 4B was prepared from BrCN-activated Sepharose 4B (Pharmacia) according to the supplier's recommendation and ω-aminooctyl-Sepharose 4B was prepared according to the published procedure (Cuatrecasas, 1970).

SDS–Polyacrylamide Gel Electrophoresis. One-dimensional SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970).

Determination of NH_2-Terminal Amino Acid Sequence. The NH_2-terminal amino acid sequence of the purified cytochrome P-450$_{11\beta}$ was determined by the automated Edman degradation on a gas-phase protein sequencer (Applied Biosystems, Model 470A) and a HPLC 8100 system of Spectra Physics.

Spectroscopic Measurements. Visible absorption spectra were recorded with a Shimadzu Model UV-240 spectrophotometer. The temperature of cell holders was controlled at 25 °C by circulating water from a thermostated water bath.

EPR measurements were carried out at 77 K or 4.2 K at X-band (9.35 GHz) microwave frequency with a home-built EPR spectrometer with 100-kHz modulation by using a Varian X-band cavity. Immersion Dewer flasks were used for the EPR measurements. The microwave frequency was calibrated with a microwave frequency counter (Takeda Riken Co., Ltd., Model TR5212). The magnetic field strength was determined by a nuclear magnetic resonance of protons in water. Accuracies of the *g* values and the hyperfine coupling constants were approximately ±0.001 and ±0.05 mT, respectively. Preparation of the ferrous–NO complexes of cytochrome P-450$_{11\beta}$ in the presence or absence of reduced adrenodoxin was done as described previously (Tsubaki et al., 1987a, 1988).

Resonance Raman spectra were obtained in the grazing incidence geometry by using a highly sensitive multichannel laser Raman system, which consists of a dry-ice-cooled silicon-intensified target (SIT) detector, a detector controller, an OMA 3 console, and a Spex 1402 double monochromator, with exciting wavelength at 406.7 or 413.1 nm from a Kr ion laser (Spectra Physics, Model 171-01) or 441.6 nm from a He–Cd laser (Liconix, Model 4240). All the wavenumbers reported herein were accurate within ±1 cm^{-1}. The sample was kept at room temperature during the measurements and the local heating by incident laser light was avoided by rotating the Raman cell.

Chemicals. 11-Deoxycorticosterone and sodium dithionite were obtained from Wako Pure Chemicals; Tween 20 was obtained from Kao-Atlas. Carbon monoxide was obtained from the following manufacturers: $^{12}C^{16}O$ from Matheson and $^{12}C^{18}O$ from Prochem. Isotopically labeled $Na^{15}NO_2$ was obtained from Merck and Co.

Results

Biochemical Properties of Cytochrome P-450$_{11\beta}$. The purified cytochrome P-450$_{11\beta}$ was fully in the ferric high-spin state as shown in Figure 1 (solid line) due to the binding of 11-deoxycorticosterone to the enzyme, which was added in the buffer throughout the purification (Suhara et al., 1978). In the ferrous–CO state, there was no formation of the "P-420"

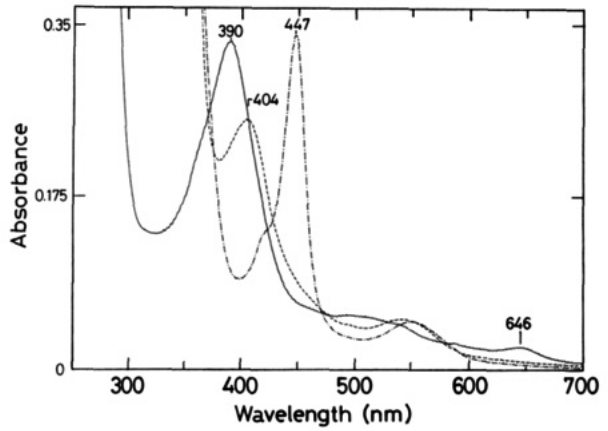

FIGURE 1: Absorption spectra of the purified cytochrome P-450$_{11\beta}$ from bovine adrenocortical mitochondria at 25 °C in the standard buffer containing 0.5% Tween 20 and 0.1% sodium cholate. Solid line, ferric high-spin state; one dotted broken line, ferrous–CO state; broken line, ferrous state.

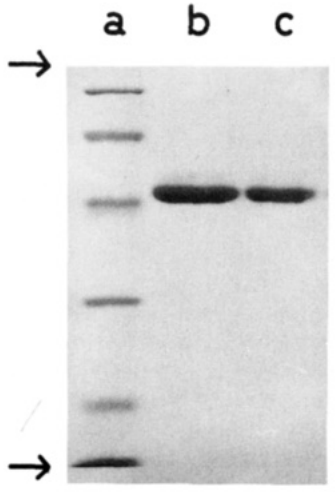

FIGURE 2: SDS–polyacrylamide gel electrophoresis of the purified cytochrome P-450$_{11\beta}$ stained with Coomassie brilliant blue R-250. Stacking gel concentration, 10%. Lane a contains standard molecular weight markers: phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100), and α-lactoalbumin (14 400). Lanes b and c contain 5.8 and 2.9 μg each of the purified cytochrome P-450$_{11\beta}$. Two arrows indicate and origin and a front of the electrophoresis.

species on the basis of the spectral examination (one dotted broken line). The Soret band peak in the ferrous–CO state was found at 447 cm^{-1}, almost identical with that of cytochrome P-450$_{scc}$ in the ferrous–CO state. In the ferrous state (broken line), the Soret peak was found at 404 nm, a much shorter wavelength than the corresponding value for cytochrome P-450$_{scc}$ (i.e., 413 nm) (Tsubaki et al., 1986b). The purified sample had a heme content of about 13 nmol of P-450/mg of protein.

The homogeneity of the purified bovine cytochrome P-450$_{11\beta}$ was assessed by one-dimensional SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) as shown in Figure 2. Its monomeric molecular weight was estimated to be about 46 000. However, when a small amount of the purified sample protein was subjected to the electrophoresis, the stained protein band was found to be actually composed of two bands (data not shown), confirming the previous observation (Ogishima et al., 1989a). It is not clear at this stage whether these two bands correspond to the heterogeneity of cytochrome P-450$_{11\beta}$ found in rat adrenal (Ohnishi et al., 1988) or not. We did not make any effort further to purify each form in its native state because of the shortage and the instability of the enzyme.

Table I: NH_2-Terminal Amino Acid Sequence of Cytochrome P-450$_{11\beta}$[a]

residue no.	primary sequence	pmol	secondary sequence	pmol
1	Gly	735	Gly	nd
2	Thr	138	Ala	136
3	Arg	278	Ala	86
4	Gly	287	Ala	194
5	Ala	349	Pro	114
6	Ala	390	Lys	82
7	Ala	612	Ala	nd
8	Pro	364	Val	146
9	Lys	278	Leu	272
10	Ala	462	Pro	249
11	Val	314	Phe	137
12	Leu	448	Glu	nd
13	Pro	484	Ala	289
14	Phe	359	Met	202
15	Glu	nd	Pro	314
16	Ala	490	Arg	297
17	Met	423	(Cys)	nd
18	Pro	398	Pro	nd
19	Arg	519	Gly	115
20	(Cys)	nd	Asn	107

[a] Amino acids in parentheses indicate unidentified residues; nd means not determined. Total amounts were indicated in the column for the primary sequence for the 1st, 7th, and 18th cycles.

The NH_2-terminal amino acid sequence of the purified cytochrome P-450$_{11\beta}$ was determined and the results are listed in Table I. The bovine cytochrome P-450$_{11\beta}$ showed two sequences, i.e., a primary sequence and a secondary sequence; the latter corresponded to a form of the enzyme from which three NH_2-terminal amino acids had been removed. It is not clear whether this observation indicates the existence of two types of processing of the enzyme for entry into the inner mitochondrial membrane or just a proteolysis that occurred during the purification. Existence of a similar form lacking three amino acids at the NH_2 terminus was reported previously for the porcine cytochrome P-450$_{11\beta}$ (Yanagibashi et al., 1986). The amino acid sequence of the primary form coincided with the one deduced from cDNA nucleotide sequence up to 20 cycles (Morohashi et al., 1987). It is unclear, however, that the heterogeneity of the enzyme on SDS–polyacrylamide gel electrophoresis is caused by the removal of three amino acids at the NH_2 terminus if one considers the difference in the molecular weight of the two forms. Indeed the existence of the third form in our preparation was clearly indicated in the presence of a fair amount of Val at the third cycle (92 pmol) and Gly at the sixth cycle (26 pmol) (Table I). But it is not convincing that the substitution of only three amino acid residues (Ala, Ser, His → Val, Gly, Arg, respectively) causes such a large difference in the electrophoretic mobility (Kirita et al., 1988; Ogishima et al., 1989a).

Resonance Raman Spectra of Cytochrome P-450$_{11\beta}$. Resonance Raman spectra in the higher frequency region (1200–1700 cm^{-1}) of cytochrome P-450$_{11\beta}$ in the ferric and ferrous states are shown in Figure 3 (left). The ν_{10} and ν_3 modes of cytochrome P-450$_{11\beta}$ in the ferric high-spin state were observed at 1629 and 1490 cm^{-1}, respectively. Both modes lie in the range of the pentacoordinated states according to Teraoka and Kitagawa (1980). The other mitochondrial enzyme, cytochrome P-450$_{scc}$, showed these bands close to the range of the hexacoordinated species rather than to that of the pentacoordinated species in the ferric high-spin cholesterol-complexed state as previously reported (Shimizu et al., 1981; Tsubaki et al., 1986a). Binding of 20(*R*),22(*R*)-dihydroxycholesterol, an intermediate metabolite during the cholesterol side-chain cleavage reaction, to the substrate-free

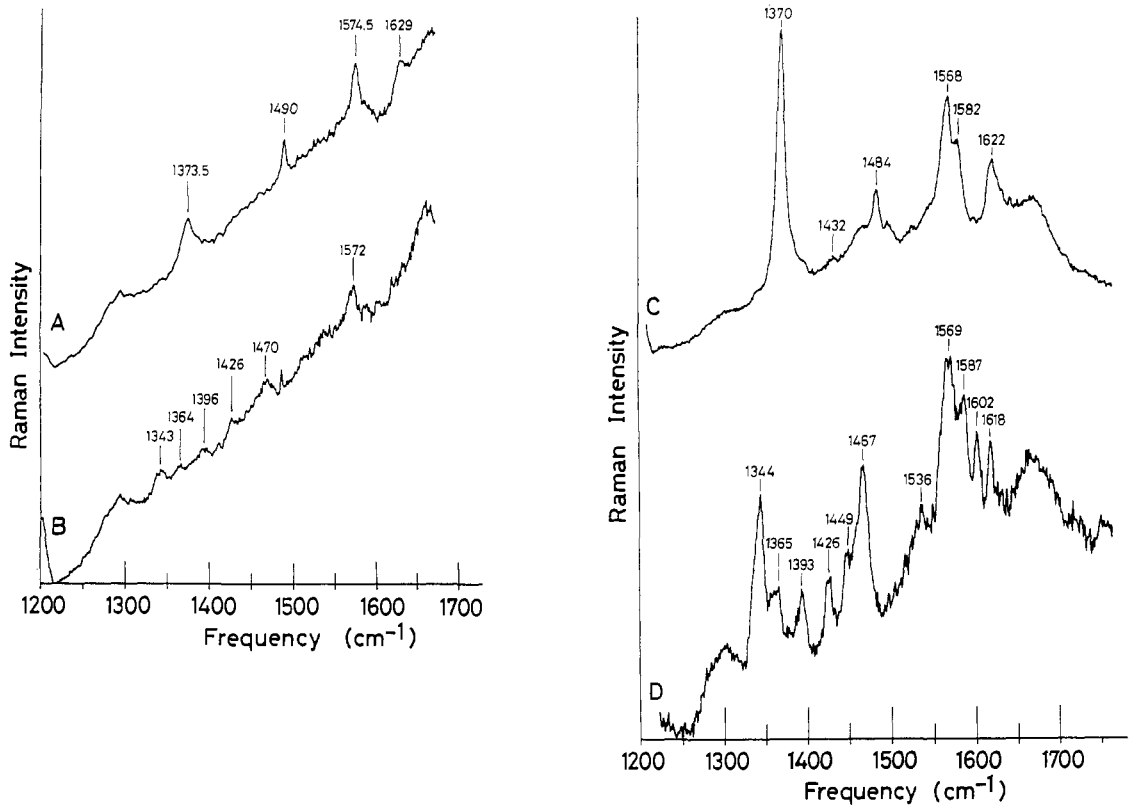

FIGURE 3: Resonance Raman spectra of the cytochrome P-450$_{11\beta}$–deoxycorticosterone and cytochrome P-450$_{scc}$–20(R),22(R)-dihydroxycholesterol complexes in the higher frequency region (1200–1700 cm^{-1}): left panel, line A, in the ferric high-spin state and line B, in the ferrous state of the cytochrome P-450$_{11\beta}$–deoxycorticosterone complex; the enzyme (~50 μM) was solubilized in the standard buffer containing 0.5% Tween 20 and 1.0% sodium cholate: right panel, line C, in the ferric high-spin state; line D, in the ferrous state of the cytochrome P-450$_{scc}$–20-(R),22(R)-dihydroxycholesterol complex; the enzyme (~50 μM) was solubilized in 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 20% glycerol. Excitation wavelength, 406.7 nm (~20 mW at the sample point); slit width, 70 μm; slit height, 2 mm.

form of cytochrome P-450$_{scc}$ caused an almost complete conversion of the ferric heme iron to the high-spin state (Tsubaki et al., 1988). Resonance Raman spectrum of this complex again showed the ν_3 and ν_{10} modes close to the range of the hexacoordinated high-spin species (1484 and 1622 cm^{-1}, respectively) as shown in Figure 3 (right).

Resonance Raman spectrum of cytochrome P-450$_{11\beta}$ in the ferrous state showed several characteristic Raman lines at 1343, 1364, 1396, 1426, 1470, and 1572 cm^{-1} (Figure 3, left). The overall spectral features were very similar to those of cytochrome P-450$_{scc}$ in the substrate-free or in the cholesterol-complexed state (Tsubaki et al., 1986a) or in the 20-(R),22(R)-dihydroxycholesterol-complexed state (Figure 3, right). The 1343-cm^{-1} band corresponds to the ν_4 mode, and this lowered frequency is unique to cytochrome P-450 in the ferrous state (Ozaki et al., 1976). Absence of a band around 1357 cm^{-1} indicates that the contribution of the "P-420" form in our preparation was negligible (Tsubaki et al., 1986a).

The ν(Fe^{2+}–CO) stretching Raman line for cytochrome P-450$_{11\beta}$ in the ferrous–CO state was identified at 481.5 cm^{-1} as shown in Figure 4B. In the ferric high-spin state (Figure 4A), there was no Raman band around this region. Upon isotopic substitution of carbon monoxide from $^{12}C^{16}O$ to $^{12}C^{18}O$, this band showed a downward frequency shift to 472 cm^{-1} (Figure 4C) very close to the expected frequency on the basis of a diatomic harmonic oscillator model. Due to the instability of the enzyme in the absence of the substrate, the ν(Fe^{2+}–CO) stretching band for the substrate-free state could not be identified.

Electron Paramagnetic Resonance Spectra of Cytochrome P-450$_{11\beta}$. The EPR spectrum of ferric cytochrome P-450$_{11\beta}$ was measured at 4.2 K and the spectrum was compared with that of cytochrome P-450$_{scc}$ complexed with 25-hydroxycholesterol as shown in Figure 5. As expected we could detect only high-spin signals at g = 7.98, 3.65, and 1.71. The g values for the ferric high-spin cytochrome P-450$_{11\beta}$ were clearly different from those of the ferric high-spin cytochrome P-450$_{scc}$–25-hydroxycholesterol complex (g = 8.06, 3.56, and 1.68). The g values of the ferric high-spin signals of cytochrome P-450$_{scc}$ were all identical within our spectral resolution, irrespective of the substrates bound to the enzyme (Table II). A closer examination of the g = 7.98 signal of cytochrome P-450$_{11\beta}$ revealed that this signal was broader than the corresponding signal of cytochrome P-450$_{scc}$ as shown in the inset of Figure 5. Indeed a half band width of the g = 7.98 signal of cytochrome P-450$_{11\beta}$ was larger than those of the corresponding signals of the ferric high-spin cytochrome P-450$_{scc}$ complexes (Table II). Further, asymmetric broadening of the g = 7.98 signal may suggest the existence of a minor species with a larger g value. This kind of the heterogeneity of the ferric high-spin signal has not been observed for the cytochrome P-450$_{scc}$–steroid complexes (Tsubaki et al., 1988; Tsubaki et al., unpublished results).

We then analyzed the EPR spectra of the ferrous–nitric oxide (NO) complex of cytochrome P-450$_{11\beta}$ in the absence and in the presence of reduced adrenodoxin. As shown in Figure 6A, the ferrous–^{14}NO complex of cytochrome P-450$_{11\beta}$ in the absence of adrenodoxin exhibited EPR signals with rhombic symmetry [g_x = 2.068, g_z = 2.000 (A_z = 2.14 mT), g_y = 1.961] at 77 K, very similar to those of the ferrous–^{14}NO complexes of cytochrome P-450$_{scc}$ in the absence of cholesterol, in the presence of 22(S)-hydroxycholesterol, or in the presence of 20(R),22(R)-dihydroxycholesterol (Table III) (Tsubaki et al., 1987a, 1988). The ferrous–^{15}NO complex of cytochrome

Table II: EPR Parameters for Ferric High-Spin and Ferrous–NO Complexes of Cytochrome P-450$_{11\beta}$ and Cytochrome P-450$_{scc}$

ferric high-spin complexes[a]	g_x (half-width)	g_y	y_z	ref
cytochrome P-450$_{11\beta}$(DOC)	7.98 (2.73 mT)	3.65	1.71	this study
cytochrome P-450$_{scc}$(Chol)	8.06 (2.11 mT)	3.55	1.68	this study
cytochrome P-450$_{scc}$(25-OH-Chol)	8.06 (2.04 mT)	3.55	1.68	this study
cytochrome P-450$_{scc}$(20,22-(OH)$_2$-Chol)	8.06 (2.24 mT)	3.55	1.68	this study
ferrous–^{14}NO complexes	g_x	g_z (A_z)	g_y	ref
cytochrome P-450$_{11\beta}$(DOC)	2.068	2.001 (2.14 mT)	1.961	this study
cytochrome P-450$_{scc}$(SF)	2.071	2.001 (2.20 mT)	1.962	*b*
cytochrome P-450$_{scc}$(Chol)	2.071	2.001 (2.20 mT)	1.962	*b*
cytochrome P-450$_{scc}$(25-OH-Chol)	2.074	1.999 (2.10 mT)	1.961	*b*
cytochrome P-450$_{scc}$(22(*S*)-OH-Chol)	2.068	2.000 (2.09 mT)	1.961	*b*
cytochrome P-450$_{scc}$(22(*R*)-OH-Chol)	2.078	2.001 (1.85 mT)	1.962	*b*
cytochrome P-450$_{scc}$(20,22-(OH)$_2$-Chol)	2.070	2.000 (2.19 mT)	1.961	*c*
cytochrome P-450$_{scc}$(20(*S*)-OH-Chol)	2.027	2.007 (1.76 mT)	1.984	*b*

[a] Abbreviations: DOC, 11-deoxycorticosterone; SF, substrate free; Chol, cholesterol; 20,22-(OH)$_2$-Chol, 20(*R*),22(*R*)-dihydroxycholesterol. [b] Tsubaki et al., 1987a. [c] Tsubaki et al., 1988.

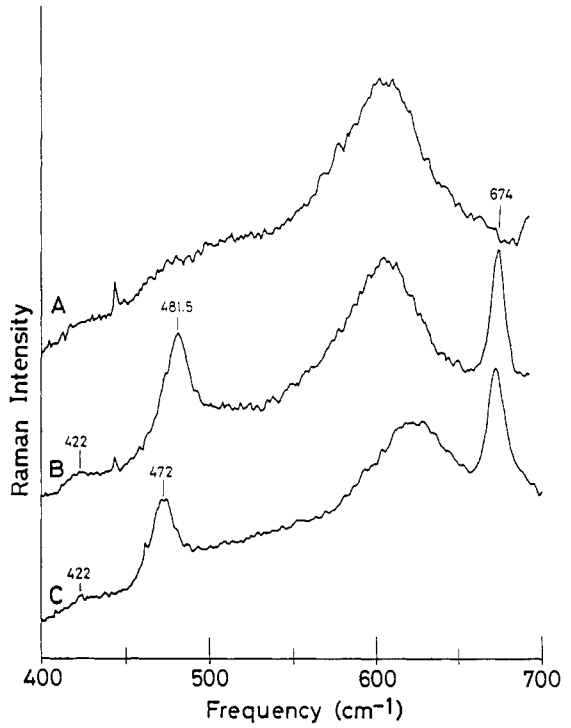

FIGURE 4: Resonance Raman identification of the ν(Fe^{2+}–CO) stretching frequency of cytochrome P-450$_{11\beta}$ in the ferrous–CO state: line A, in the ferric high-spin state; line B, in the ^{12}C^{16}O-complexed state; line C, in the ^{12}C^{18}O-complexed state. Other conditions were the same as in Figure 3. The Raman bands at 674 and 422 cm^{-1} are from porphyrin ring modes, and broad features around 600 cm^{-1} are due to the Raman scattering from the glass of the Raman cell.

P-450$_{11\beta}$ showed its EPR parameters [g_x = 2.068 (A_x = 1.01 mT), g_z = 2.000 (A_z = 3.01 mT), g_y = 1.961] very similar to those of the ^{14}NO complex except for the hyperfine splitting due to nitrogen nuclei (^{14}NO versus ^{15}NO) (Figure 6C and Table II). There was no drastic EPR spectral change upon addition of reduced adrenodoxin to the ferrous–^{14}NO and –^{15}NO complexes of cytochrome P-450$_{11\beta}$ except for the appearance of signals originating from reduced adrenodoxin itself (g = 2.021 and 1.933) as shown in Figure 6B,D. However, for some preparations, it was observed that a new EPR species with less rhombic (or quasiaxial) symmetry characterized by a g = 2.034 signal developed gradually during the incubation with reduced adrenodoxin at 0 °C, indicating the generation of a species having the polar surroundings of the ferrous–NO heme moiety (spectra not shown). This phenomenon was similar to that of the ferrous–NO complex of cytochrome P-450$_{scc}$ in the presence of cholesterol (Tsubaki et al., 1988),

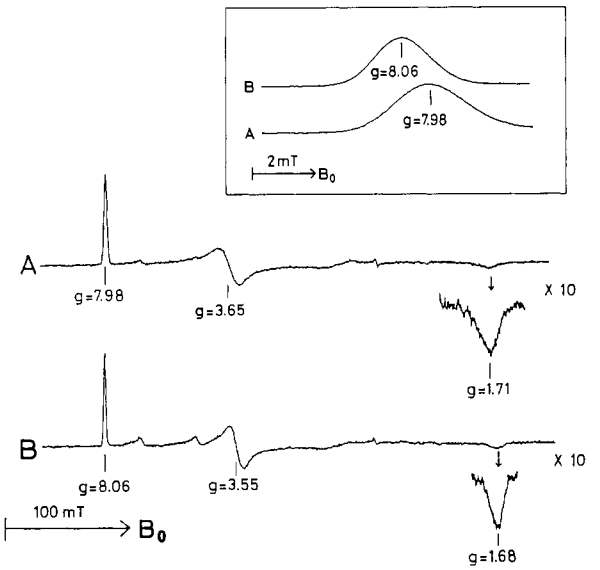

FIGURE 5: Electron paramagnetic resonance (EPR) spectra of the cytochrome P-450$_{11\beta}$–deoxycorticosterone complex in the ferric high-spin state (A) in comparison with that of the cytochrome P-450$_{scc}$–25-hydroxycholesterol complex in the ferric high-spin state (B) at 4.2 K. Inset shows enlargement of the g = 8 region. EPR conditions: microwave power, 5 mW; modulation amplitude, 2 G; microwave frequency, 9.35 GHz. Cytochrome P-450$_{11\beta}$ (~300 μM) was solubilized in the standard buffer containing 0.5% Tween 20 and 1.0% sodium cholate, whereas cytochrome P-450$_{scc}$ was in 10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. Other conditions were described under Experimental Procedures.

Table III: Resonance Raman Frequencies of Structure-Sensitive Bands of Ferric High-Spin Cytochrome P-450s

ferric high-spin complexes[a]	ν_3	ν_{10}	coordination no.	ref
cytochrome P-450$_{scc}$(Chol)	1485	1617	6	*b*
cytochrome P-450$_{scc}$(Chol)	1485	1620	6	*c*
cytochrome P-450$_{scc}$(20,22-(OH)$_2$-Chol)	1484	1622	6	this study
cytochrome P-450$_{cam}$(camphor)	1488	1623	5	*d*
cytochrome P-450$_{LM4}$(SF)	1487	1627	5	*e*
cytochrome P-450$_{LM4}$(SF)	1488	1627	5	*f*
cytochrome P-450$_{LM4}$(NF)	1488	1625	5	*e*
cytochrome P-450$_{LM4}$(NF)	1488	1624	5	*f*
cytochrome P-450$_{LM2}$(BP)	1488		5	*g*
cytochrome P-450$_{rat,LM}$	1490		5	*h*
cytochrome P-450$_{11\beta}$(DOC)	1490	1629	5	this study

[a] Abbreviations: NF, β-naphthoflavone; BP, benzphetamine. [b] Shimizu et al., 1981. [c] Tsubaki et al., 1986a. [d] Champion et al., 1978. [e] Hildebrandt et al., 1989a. [f] Hildebrandt et al., 1989b. [g] Hildebrandt et al., 1988. [h] Anzenbacher et al., 1980.

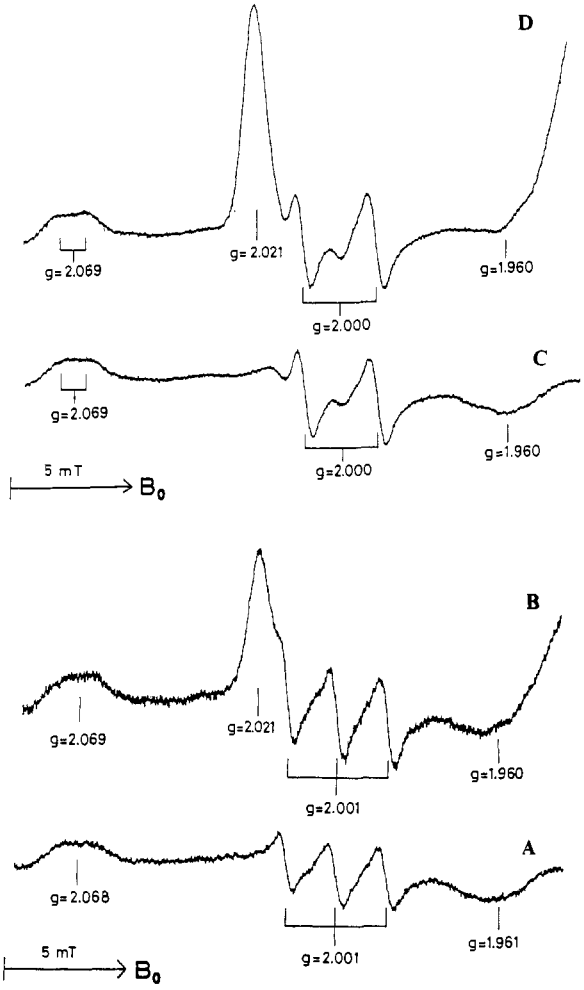

FIGURE 6: Electron paramagnetic resonance spectra of the ferrous-^{14}NO (lines A and B) and -^{15}NO (lines B and D) complexes of cytochrome P-450$_{11\beta}$ in the presence of (lines B and D) and in the absence of (lines A and C) reduced adrenodoxin. Other conditions are the same as in Figure 5.

where, however, there was always a time-dependent spectral change of the EPR signals with rhombic symmetry (i.e., g = 2.071 signal shifted to g = 2.075) simultaneously.

Discussion

Ferric High-Spin States. In the present study we observed clear differences between cytochrome P-450$_{11\beta}$ and cytochrome P-450$_{scc}$ in the ferric high-spin state (i.e., in the substrate-bound state). These differences seem to originate from the different coordination configuration of the ferric heme iron in these enzymes.

Resonance Raman spectroscopy is an indispensable technique to detect a small structural change of the heme moiety. Because the vibrational modes of the heme are very sensitive to its core size, several Raman bands such as the ν_3 and ν_{10} modes reflect the spin state and the coordination number of the heme iron. For the ferric high-spin species, it is known that the ν_3 mode (polarized, maximum intensity with Soret band excitation) is at the high end of the range for the pentacoordinated species (1488–1496 cm^{-1}) but at the low end for the hexacoordinated complexes (1475–1485 cm^{-1}) (Shimizu et al., 1981; Anzenbacher et al., 1981). The ν_{10} mode (depolarized, maximal intensity with Q band excitation) also shows a similar relationship; 1628–1633 cm^{-1} for the pentacoordinated and 1608–1622 cm^{-1} for the hexacoordinated ferric high-spin complexes (Teraoka et al., 1980; Shimizu et al., 1981); although the above classification on the ν_{10} mode is not in complete accordance with that of Sharma et al. (1989).

On the basis of the ν_3 and ν_{10} Raman frequencies, however, the ferric cytochrome P-450$_{11\beta}$–deoxycorticosterone complex can be classified clearly as a pentacoordinated species. The adaptation to the pentacoordinated high-spin state upon binding of the substrate or its analogue seems to be common for microsomal cytochrome P-450 (Table III). On the contrary, the ferric cytochrome P-450$_{scc}$ complexed with 20-(R),22(R)-dihydroxycholesterol or cholesterol seems to adopt a hexacoordinated state (or, at least, an intermediate configuration between the pentacoordinated and the hexacoordinated high-spin states) (Table III).

It is known that a water molecule (or hydroxyl ion) binds to the ferric heme iron of cytochrome P-450$_{cam}$ in the absence of substrate and is excluded from the heme center in the substrate (camphor) complex of cytochrome P-450$_{cam}$ on the basis of X-ray crystallographic studies (Poulos et al., 1985, 1987). Upon the exclusion of a water molecule, the ferric heme iron assumes the pentacoordinated state and changes the spin state from low to high, almost completely. Results from NMR measurements on the relaxation rate of water protons of cytochrome P-450$_{cam}$ solution in the presence or absence of the substrate (camphor) seem to support this conclusion (Griffin & Peterson, 1975; Philson et al., 1979). According to their studies, the minimum distance for the closest approach of solvent protons to the heme iron was estimated to be 2.0–2.6 Å for the substrate-free state and 5.9–9.2 Å for camphor-bound state, respectively (Griffin & Peterson, 1975; Philson et al., 1979). The resonance Raman data for cytochrome P-450$_{cam}$(camphor) in solution (ν_3 = 1488 cm^{-1}; in the range for the pentacoordinated state) is also consistent with this view (Champion et al., 1978). A similar NMR study on the relaxation rate of water protons of cytochrome P-450$_{scc}$ solution (Jacobs et al., 1987) showed that water protons have rapid access to the regions near the active site (heme iron) for several steroid complexes of cytochrome P-450$_{scc}$. Even for the cholesterol complex, the minimum distance for the closest approach of solvent protons to the heme iron was estimated to be 3.9 Å, only slightly larger than the value for the substrate-free state (2.5 Å).

Considering these results, the present resonance Raman data seem to suggest that the active site of cytochrome P-450$_{scc}$ is more open to the access of the solvent water molecule than those of cytochrome P-450$_{11\beta}$, cytochrome P-450$_{cam}$, or other microsomal cytochrome P-450s in the ferric high-spin state. If we adopt this assumption, it is easily understood why the binding of cholesterol to the substrate binding site, which is generally considered to be very hydrophobic, of cytochrome P-450$_{scc}$ is so weak and slow. Indeed, much more hydrophilic substrates, such as 20(S)-, 22(R)-, and 25-hydroxycholesterols and 20(R),22(R)-dihydroxycholesterol have much higher binding affinities toward cytochrome P-450$_{scc}$ than cholesterol does. It may be further suggested that for the side-chain cleavage reaction to occur properly the environment of the active site of cytochrome P-450$_{scc}$ should be rather hydrophilic. In this context the suggestion made by Jacobs et al. (1987) that the solvent water molecule near the active site may provide the protons required for the monooxygenation and the C20–C22 bond scission is very interesting.

Presently we do not have enough data to correlate between the coordination number (or configuration) of the heme iron and the EPR spectrum in the ferric high-spin state. But in this study we could observe a clear difference between cytochrome P-450$_{11\beta}$ and cytochrome P-450$_{scc}$ in the EPR spectra

for the ferric high-spin state. Therefore it is very likely that this difference is due to the difference in the coordination number of the ferric heme iron. The nature of the broadening (or the heterogeneity) of the ferric high-spin $g = 7.98$ signal of cytochrome P-450$_{11\beta}$ is not clear. This can be caused by the local heterogeneity of the active center (coordination number of the heme iron or stereochemical structure) or by the molecular multiplicity of cytochrome P-450$_{11\beta}$ itself.

Previously Brownie et al. (1973) reported that the low-field EPR signal of rat adrenal mitochondria consists of two components, one appearing at $g = 7.9$ and the other at $g = 8.2$. The magnitude of the signal at $g = 7.9$ was greatly enhanced when 11-deoxycorticosterone was added. On the other hand, addition of the high concentration of pregnenolone caused a decrease in signal intensity at $g = 8.2$. Therefore they interpreted these to mean that the signal at $g = 7.9$ was that of cytochrome P-450$_{11\beta}$ in the high-spin form (i.e., substrate-bound form), whereas the signal at $g = 8.2$ was that of cytochrome P-450$_{scc}$ in its high-spin form. Later Jefcoate et al. (1976) examined bovine adrenal mitochondria by EPR spectroscopy and observed two distinct high-spin forms of cytochrome P-450s also. The predominant high-spin species ($g = 8.2$) was decreased by addition of 20(S)-hydroxycholesterol and was increased by addition of cholesterol. The minor high-spin species ($g = 8.1$) was increased by addition of 11-deoxycorticosterone but was decreased by metyrapone. The two forms were, accordingly, assigned to cytochrome P-450$_{scc}$ and cytochrome P-450$_{11\beta}$, respectively. Our present EPR results confirmed these assignments unequivocally.

Ferrous Ligated States. Contrary to the results for the ferric high-spin state, we have obtained some evidence suggesting a close similarity of the stereochemistry of the active site of cytochrome P-450$_{11\beta}$ to that of cytochrome P-450$_{scc}$ in the ferrous ligated states (i.e., ferrous–CO and ferrous–NO states) and probably in the ferrous state as well.

The identified $\nu(Fe^{2+}–CO)$ frequency of cytochrome P-450$_{11\beta}$ (481.5 cm^{-1}) is very close to the corresponding frequency of the cytochrome P-450$_{scc}$–cholesterol complex (483 cm^{-1}) (Tsubaki et al., 1986a). It must be noted that cytochrome P-450$_{11\beta}$ used was in the 11-deoxycorticosterone-complexed state and the $\nu(Fe^{2+}–CO)$ frequency of cytochrome P-450$_{scc}$ varied from 477 cm^{-1} (substrate-free) to 487 cm^{-1} (22(S)-hydroxycholesterol) depending on the substrate bound (Tsubaki & Ichikawa, 1985; Tsubaki et al., 1986a). In this context the immediate environments of the ferrous heme–CO moiety of the cytochrome P-450$_{11\beta}$–deoxycorticosterone complex and the cytochrome P-450$_{scc}$–cholesterol complex may be very similar to each other. These rather lower $\nu(Fe^{2+}–CO)$ stretching frequencies are common to various cytochrome P-450s so far reported (Tsubaki & Ichikawa, 1985; Uno et al., 1985) and showed remarkable differences from other ferrous–CO hemoproteins with a nitrogenous fifth ligand as previously reported (Tsubaki et al., 1982; Tsubaki & Ichikawa, 1985). The EPR spectra of the ferrous–NO complexes in the presence or absence of reduced adrenodoxin were also very similar between these two cytochrome P-450s as noted in the Results, indicating similarities in the binding configuration and in the immediate environment of bound nitric oxide between these two enzymes. In the ferrous state, there is no water coordination to the ferrous heme iron, adopting the pentacoordinated structure. Therefore it is reasonable to see no significant difference between these two enzymes in the resonance Raman spectra. (The reason for the difference in Soret peak wavelength in the ferrous state between cytochrome P-450$_{11\beta}$ and cytochrome P-450$_{scc}$ is currently unknown.)

Recently we have identified the adrenodoxin-binding site of cytochrome P-450$_{scc}$ for the first time (Tsubaki et al., 1989a). This site is located at the proximal side of the heme corresponding to the K helix of cytochrome P-450$_{cam}$ and is characterized by a well-conserved sequence among mitochondrial cytochrome P-450s [including cytochrome P-450$_{11\beta}$ and cytochrome P-450$_{sterol\ 26\text{-}hydroxylase}$ (Anderson et al., 1989)]. We have already investigated the influence of adrenodoxin binding to this site on the heme moiety of cytochrome P-450$_{scc}$ in various states (Tsubaki et al., 1986a, 1988, 1989b). Considerable influence was observed upon the binding of reduced adrenodoxin to the ferrous–NO complex of cytochrome P-450$_{scc}$ (Tsubaki et al., 1988). It is noticeable that such an effect was observed only in the cholesterol-complexed state (and marginally in the substrate-free state). We consider that the change of the EPR spectra of the ferrous–NO complex of cytochrome P-450$_{scc}$ upon the binding of reduced adrenodoxin in the presence of cholesterol (i.e., from $g = 2.071$ to $g = 2.075$) is due to the uniqueness of cholesterol as substrate as suggested previously (Tsubaki et al., 1988). For other steroid complexes we could not detect any significant influences. In this context it may be reasonable that the binding of reduced adrenodoxin caused no apparent effect on the EPR spectra of the ferrous–NO complexes of cytochrome P-450$_{11\beta}$ in the presence of 11-deoxycorticosterone. To clarify the molecular mechanism of the cytochrome P-450$_{11\beta}$ catalyzed reaction, more detailed and systematic studies including the comparison with cytochrome P-450$_{scc}$ are required.

In conclusion, in the present study, we have obtained several pieces of evidence indicating that the stereochemistry of the active site of cytochrome P-450$_{11\beta}$ bears a close resemblance to that of cytochrome P-450$_{scc}$ in the ferrous ligated states (ferrous–CO and ferrous–NO states) but not in the substrate-bound ferric high-spin state.

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