Effects of Cholesterol Side-Chain Groups and Adrenodoxin Binding on the Vibrational Modes of Carbon Monoxide Bound to Cytochrome P-450$_{scc}$: Implications of the Productive and Nonproductive Substrate Bindings

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ABSTRACT: Effects of the bindings of cholesterol and its hydroxylated analogues on the Fe–CO stretching and the C–O stretching vibrations of cytochrome P-450$_{scc}$–CO complex were examined by resonance Raman and FT-IR spectroscopies to reveal the spatial relationship between the steroid side-chain groups and the heme-bound C–O moiety at the active center. These C–O and Fe–CO vibrations exhibited considerable variations depending on the steroids used; however, analyses on the ν_{Fe-CO} vs ν_{C-O} plot for cytochrome P-450$_{scc}$ indicated the absence of the negative correlation between these two vibrations, which is common among various Fe^{2+}–porphyrin–CO complexes having imidazole ligands. Rather, we noticed the existence of two groups depending on substrates, the one exhibiting C–O infrared absorption bands in the region from 1930 to 1940 cm^{-1} and higher enzymatic turnover numbers in the reconstituted enzymatic systems and the other exhibiting C–O infrared absorption bands in the region above 1945 cm^{-1} and lower enzymatic turnover numbers. Thus, the former substrate group is likely to be fitted into the substrate binding site in the efficient "productive substrate binding" structure, whereas the latter group may be bound to the enzyme in the structure not suitable for the efficient enzymatic reaction ("nonproductive substrate binding" conformation). Among the steroids examined, 22(*R*)-hydroxycholesterol (a physiological intermediate in the cholesterol side-chain cleavage reaction) was unique since its binding to cytochrome P-450$_{scc}$ caused the appearance of two C–O infrared bands, one at 1934 cm^{-1} suggestive of the productive substrate binding and the other at 1951 cm^{-1} indicative of the nonproductive substrate binding. Further, the two C–O infrared bands showed a temperature-dependent intensity change (in the region from 2 to 30 °C), suggesting that at higher temperature the productive substrate binding is favored. Binding of reduced adenodoxin interferes with the CO binding only for the species having nonproductive substrate binding structure.

Side-chain cleavage reaction of cholesterol to pregnenolone is the rate-limiting step in steroid hormone biosynthesis at adrenal cortex and is catalyzed by mitochondrial cytochrome P-450$_{scc}$ with its NADPH-specific electron-transport system (NADPH-adrenodoxin reductase and adrenodoxin) (Lambeth et al., 1979, 1982). The cholesterol side-chain cleavage reaction involves three consecutive hydroxylation steps. Each step requires two electrons supplied from adrenodoxin and one molecule of oxygen (Shikita & Hall, 1974; Jefcoate, 1986).

Adrenodoxin, an iron–sulfur protein, forms 1:1 complexes with both NADPH-adrenodoxin reductase and cytochrome P-450$_{scc}$. During each step of the electron-transfer reaction, the complexes appear to function sequentially by "adrenodoxin shuttle mechanism" (Lambeth et al., 1980; Lambeth & Pember, 1983); i.e., adrenodoxin first forms its complex with and accepts an electron from NADPH-adrenodoxin reductase, then dissociates, and finally forms a 1:1 complex with and transfers an electron to cytochrome P-450$_{scc}$. The adrenodoxin-binding site of cytochrome P-450$_{scc}$ has been revealed by specific chemical modification techniques and is believed to be formed with K and L helices (by cytochrome P-450$_{cam}$ notation) at the proximal side of the heme iron (Tsubaki et al., 1989a; Tuls et al., 1989), and the positively charged residues (Lys and Arg) at this site may have important role in the interaction with negatively charged adrenodoxin.

The first hydroxylation of the cholesterol side chain occurs at the 22(*R*) position to yield 22(*R*)-OH-cholesterol and the second at the 20(*S*) position to give 20(*R*),22(*R*)-dihydroxycholesterol. The final step results in an oxidative cleavage of the diol forming pregnenolone and isocaproic aldehyde (Burnstein et al., 1975; Larroque et al., 1981; Hume et al., 1984). It has been suggested that the oxygen molecule bound to the ferrous heme and the side-chain group of cholesterol at the substrate-binding site have a strict stereochemical interaction to advance the successive hydroxylation steps properly on the basis of the results obtained by various techniques (Tsubaki et al., 1987a).

Resonance Raman and infrared spectroscopies of hemoproteins can provide unique information on the structure of the heme moiety (Tsubaki et al., 1982; Bangcharoenpaurpong et al., 1986; Hu & Kincaid, 1991a,b; Hu et al., 1991; Egawa et al., 1991). In recent resonance Raman studies on ferrous cytochrome P-450$_{scc}$–CO complex, we detected a Fe–CO stretching vibration resonance-enhanced at 477 cm^{-1} upon Soret excitation (Tsubaki & Ichikawa, 1985), and this vibration suffered considerable influences upon steroid binding (Tsubaki et al., 1986, 1987b), indicating the existence of a strong steric interaction between heme-bound C–O and the steroid side-chain group. For cytochrome P-450$_{cam}$–CO complex extensive resonance Raman studies have been done, and those studies also suggest the existence of such steric

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interaction between heme-bound ligand and substrate at the substrate-binding site (Uno et al., 1985; Tsuboi, 1988). Recently such works have been extended to cytochrome P-$450_{11\beta}$, another steroidogenic mitochondrial enzyme in adrenal cortex (Tsubaki et al., 1990), and similarity to and difference from cytochrome P-450_{scc} were discussed.

On the other hand, despite its higher sensitivity than the resonance Raman scattering, only a few works have been done for cytochromes P-450 by infrared spectroscopy, except cytochrome P-450_{cam}. This is due to the requirement of relatively high concentration of the enzyme (in submillimolar order on heme basis) for the measurement of the infrared spectrum.

In the present study we have further extended our resonance Raman study on cytochrome P-450_{scc}, in combination with Fourier-transform infrared spectroscopy technique, to obtain further insights on the interactions of cholesterol and hydroxycholesterols with the heme moiety of cytochrome P-450_{scc} in the presence or in the absence of adrenodoxin, the physiological electron donor to mitochondrial cytochromes P-450. Understanding such interactions is essential to clarify the mechanism of enzymatic reactions such as the dioxygen activation and the site-specific hydroxylation performed by cytochrome P-450.

MATERIALS AND METHODS

Cytochrome P-450_{scc}, cytochrome P-$450_{11\beta}$, and adrenodoxin were purified from bovine adrenal cortex mitochondria as previously described (Tsubaki et al., 1986, 1990). Cytochrome P-$450_{11\beta}$ was prepared as the deoxycorticosterone (DOC)-bound form. Endogenous cholesterol and catalytic intermediates during the cholesterol side-chain cleavage reaction bound to cytochrome P-450_{scc} were removed during purification (Tsubaki et al., 1986), and nonionic detergent, Emulgen 913, was also removed during the purification procedure as previously described (Tsubaki et al., 1986).

The excitation wavelength used for resonance Raman measurements was 441.6 nm from a He–Cd laser (Liconix Model 4240). Resonance Raman spectra were obtained in the grazing incidence geometry by using a highly sensitive multichannel laser Raman system, which has a dry-ice-cooled silicon-intensified target (SIT) detector, a detector controller, an OMA 3 console, and a Spex 1402 double monochromator. The samples were kept at room temperature during the measurements, and local heating by incident laser light was avoided by rotating the Raman cell. Frequencies determined by Raman spectroscopy are accurate to ± 1.0 cm^{-1}.

The infrared spectra were recorded with a Perkin-Elmer Model 1850 Fourier-transform infrared spectrophotometer interfaced to a Perkin-Elmer 7700 computer, and this system was under the control of CDS-3 application software for data acquisition and manipulation. The infrared spectrophotometer was run in a double-beam mode. The reference IR cell contained the P-450_{scc} solution in oxidized state in the same concentration, or it is otherwise indicated. The temperature of the IR cells was maintained at 4 °C (or otherwise indicated) by circulating water from a temperature-controlled water bath through cell holders. Nominal spectral resolution at 2.0 cm^{-1} was used to record the infrared spectra. Infrared spectra with good signal-to-noise ratio were obtained by using a high enzyme concentration (0.3–0.5 mM) combined with the spectral accumulation via Fourier-transformation of the time-based interferograms into frequency-related infrared spectral data. Typically 200–400 cycles of spectral accumulations were required to obtain spectra of good quality. Data points were collected every 0.1 cm^{-1}. Frequencies determined by infrared spectroscopy are accurate to ± 0.2 cm^{-1}. Slope correction, if necessary, was made by subtracting the automatically (or manually) generated parabolic curve from the spectra. There was no additional averaging or smoothing of the data. The C–O infrared band was analyzed by spectral deconvolution using the "fitg" program that contains QUANT 3 program supplied from Perkin-Elmer (Tsubaki and Yoshikawa, unpublished data), in which peak frequencies (in cm^{-1}), half-band widths (in cm^{-1}), and spectral shape (an arbitrary linear combination of Gaussian and Lorentzian components) of the component bands were specified manually for the calculation. The "fitg" program gives the best fitted contribution of each component band by a least-squares curve fitting approach, which can use information from the entire spectrum rather than from a few selected frequencies. This program also can locate the baseline automatically by incorporating a sloping (or curved, if desired) baseline as an additional component in the fitting procedure. The closeness of the fitted bands to the actual spectrum can be judged by the flatness of the residual, i.e., the difference spectrum, obtained by subtracting the sum of the theoretical curves from the observed spectrum. The integrated absorption intensities were calculated with the Perkin-Elmer 7700 computer using a built-in program in CDS-3.

RESULTS

Effects of Steroid Side-Chain Group and Adrenodoxin Bindings on the Fe–CO Stretching Frequency. In previous series of papers we have shown that steroid bindings to the substrate-binding site of cytochrome P-450_{scc} caused a substantial variation of the bound Fe–CO stretching vibration (Tsubaki et al., 1986, 1987). More accurate resonance Raman measurements were done in the present study, and the previous results were confirmed as described below. In addition we have tried to see the effect of reduced adrenodoxin binding to cytochrome P-450_{scc} on the Fe–CO stretching frequency for the first time. Only parts of the results are shown in Figure 1.

In the substrate-free (SF) state, the Fe–CO stretching vibration appears at 476 cm^{-1}, very close to the value as previously reported (Tsubaki & Ichikawa, 1985), and the reduced adrenodoxin binding caused no influence on the frequency at all (Figure 1A). In the cholesterol-bound form, the Fe–CO stretching vibration was found at 482 cm^{-1} as previously reported (Tsubaki et al., 1986) and the binding of reduced adrenodoxin to this form caused a slight upward shift to 483.5 cm^{-1} and the broadening of the Fe–CO stretching band (Figure 1B). For the 22(*S*)-OH-cholesterol bound form, the Fe–CO stretching band was shown at 487 cm^{-1} as previously reported (Tsubaki et al., 1987) and the binding of reduced adrenodoxin again caused a slight upward shift to 488 cm^{-1} (Figure 1C). Results in Figure 1 and other measurements in the present and previous studies are summarized in Table I. In summary, (1) there is a somewhat large variation in the Fe–CO frequencies ranging from 476 to 487 cm^{-1} depending on the nature of the steroid side-chain group, and (2) the binding of reduced adrenodoxin seemed to have a tendency to cause a slight upward shift of the Fe–CO stretching vibration.

Effects of Steroid Side-Chain Group and Adrenodoxin Binding on the Bound C–O Stretching Vibration. The heme-bound C–O stretching infrared band of cytochrome P-450_{scc} was found at 1952.5 cm^{-1} for the substrate-free (SF) form (Figure 2A). This value is consistent with the reported value of 1953 cm^{-1} for the substrate-free form of cytochrome P-450_{scc} obtained by resonance Raman spectroscopy (Tsubaki

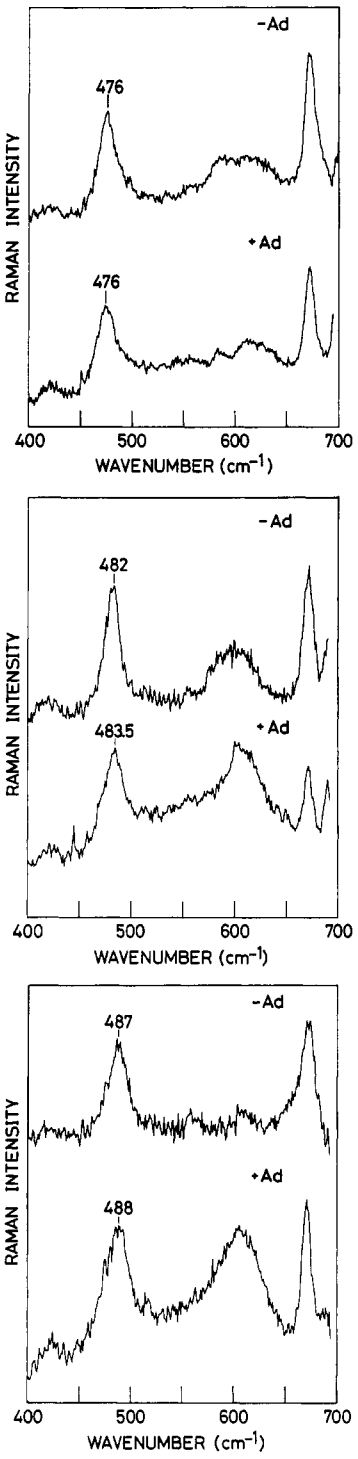

FIGURE 1: Effects of steroid side-chain group and reduced adrenodoxin binding to cytochrome P-450$_{scc}$ on the Fe–CO stretching vibration. (A, Top) Substrate-free form; (B, middle) cholesterol-bound form; (C, bottom) 22(*S*)-OH-cholesterol-bound form. Cytochrome P-450$_{scc}$–CO complex and adrenodoxin concentrations were 50 and 60 μM, respectively, in 10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. Spectra were recorded at room temperature with spinning. Conditions: excitation wavelength, 441.6 nm; laser power at sample point, 5 mW; slit width, 150 μm; slit height, 2 cm. The spectra were the average of 100 scans.

& Ichikawa, 1985). The effect of the reduced adrenodoxin binding to cytochrome P-450$_{scc}$ on the bound C–O stretching vibration was also examined for the first time. There was a slight downward shift by 1.0 cm^{-1} to 1951.5 cm^{-1} and a slight broadening of the band (Figure 2A). In cholesterol-bound form the bound C–O stretching frequency was found at 1953.7 cm^{-1}, and this band showed a 1.8 cm^{-1} downward shift upon

Table I: Effects of Reduced Adrenodoxin Binding of the ν_{C-O} and ν_{Fe-CO} Stretching Vibrations of Cytochrome P-450$_{scc}$ and Cytochrome P-450$_{11\beta}$ Complexed with Various Steroids

	adrenodoxin	ν_{C-O} (cm^{-1})	$\Delta\nu_{1/2}$ (cm^{-1})	ν_{Fe-CO} (ref)[a] (cm^{-1})
cytochrome P-450$_{scc}$				
SF[b]	–	1952.5	12.6	476 (477)[a]
SF	+	1951.5	12.4	476
cholesterol	–	1953.7	12.9	482 (483)[a]
cholesterol	+	1951.9	12.5	483.5 (483)[a]
25-OH-cholesterol	–	1954.7	9.9	484 (483)[a]
25-OH-cholesterol	+	1954.6	10.4	484
22(*R*)-OH-cholesterol	–	1951.8	12.6	479
		1934.5	11.6	
22(*R*)-OH-cholesterol	+	1951.7	12.6	480
		1933.4	12.6	
22(*S*)-OH-cholesterol	–	1946.8	10.4	487 (487)[a]
22(*S*)-OH-cholesterol	+	1946.2	10.7	488
20(*S*)-OH-cholesterol	–	1949.5	17.0	nm[c] (478)[a]
20(*S*)-OH-cholesterol	+	1946.2	17.7	nm
20,22-(OH)$_2$-cholesterol	–	nd[d]	nd	nd (nd)[a]
20,22-(OH)$_2$-cholesterol	+	1937.2	13.6	482
22-ketocholesterol	–	1950.6	13.0	nm (478)[a]
22-ketocholesterol	+	1949.0	13.2	nm
cytochrome P-450$_{11\beta}$				
DOC[e]	–	1937.3	8.8	nm (481.5)[b]
DOC	+	1937.2	9.1	nm

[a] Data obtained from previous studies: a, Tsubaki et al. (1986); b, Tsubaki et al. (1990). [b] SF, substrate free. [c] nm, not measured. [d] nd, not detected. [e] DOC, deoxycorticosterone.

binding of reduced adrenodoxin (Figure 2B). For the 22(*S*)-OH-cholesterol-bound form the bound C–O stretching vibration was seen at 1946.8 cm^{-1}, and this C–O infrared band shifted to 1946.2 cm^{-1} upon (reduced) adrenodoxin binding (Figure 2C). For the 20(*S*)-OH-cholesterol-bound form the C–O stretching frequency was found at 1949.5 cm^{-1}, and the binding of reduced adrenodoxin to this form caused a 3.3-cm^{-1} downward shift to 1946.2 cm^{-1} (Figure 2D). Formation of the ferrous–CO complex is very difficult when 20(*R*),22(*R*)-dihydroxycholesterol, the second physiological intermediate in the cholesterol side-chain cleavage, is bound to cytochrome P-450$_{scc}$ (Tsubaki et al., 1988). Addition of (reduced) adrenodoxin made the formation of the ferrous–CO complex much easier, but still a major part of the enzyme was in the reduced five-coordinated form on the basis of the visible absorption spectrum. A weak C–O infrared band at 1937.2 cm^{-1} and a shoulder around 1950 cm^{-1} were observed in the infrared spectrum (spectrum not shown).

The effect of addition of the first physiological intermediate in the side-chain cleavage reaction, 22(*R*)-OH-cholesterol, will be described in detail in the next section. Data for other steroid complexes (25-OH-cholesterol, 22-ketocholesterol; spectra not shown) are also included in Table I.

C–O Infrared Band of Cytochrome P-450$_{scc}$–22(R)-Hydroxycholesterol in the Presence or Absence of Reduced Adrenodoxin. The formation of ferrous–CO complex of cytochrome P-450$_{scc}$ in the presence of 22(*R*)-OH-cholesterol was very slow when sodium dithionite was used as a reductant, and this might be due to the interference of CO binding by the side-chain group of the steroid (see Discussion). Addition of adrenodoxin (20% mole excess over cytochrome P-450$_{scc}$) accelerated the formation of the ferrous–CO complex. The time course of the ferrous–CO complex formation in the presence or absence of (reduced) adrenodoxin was pursued by infrared spectroscopy. The most striking observation was the appearance of two sharp C–O infrared bands around 1952 and 1934 cm^{-1}. In the absence of adrenodoxin, the two infrared bands at 1952.4 and 1934.3 cm^{-1} developed

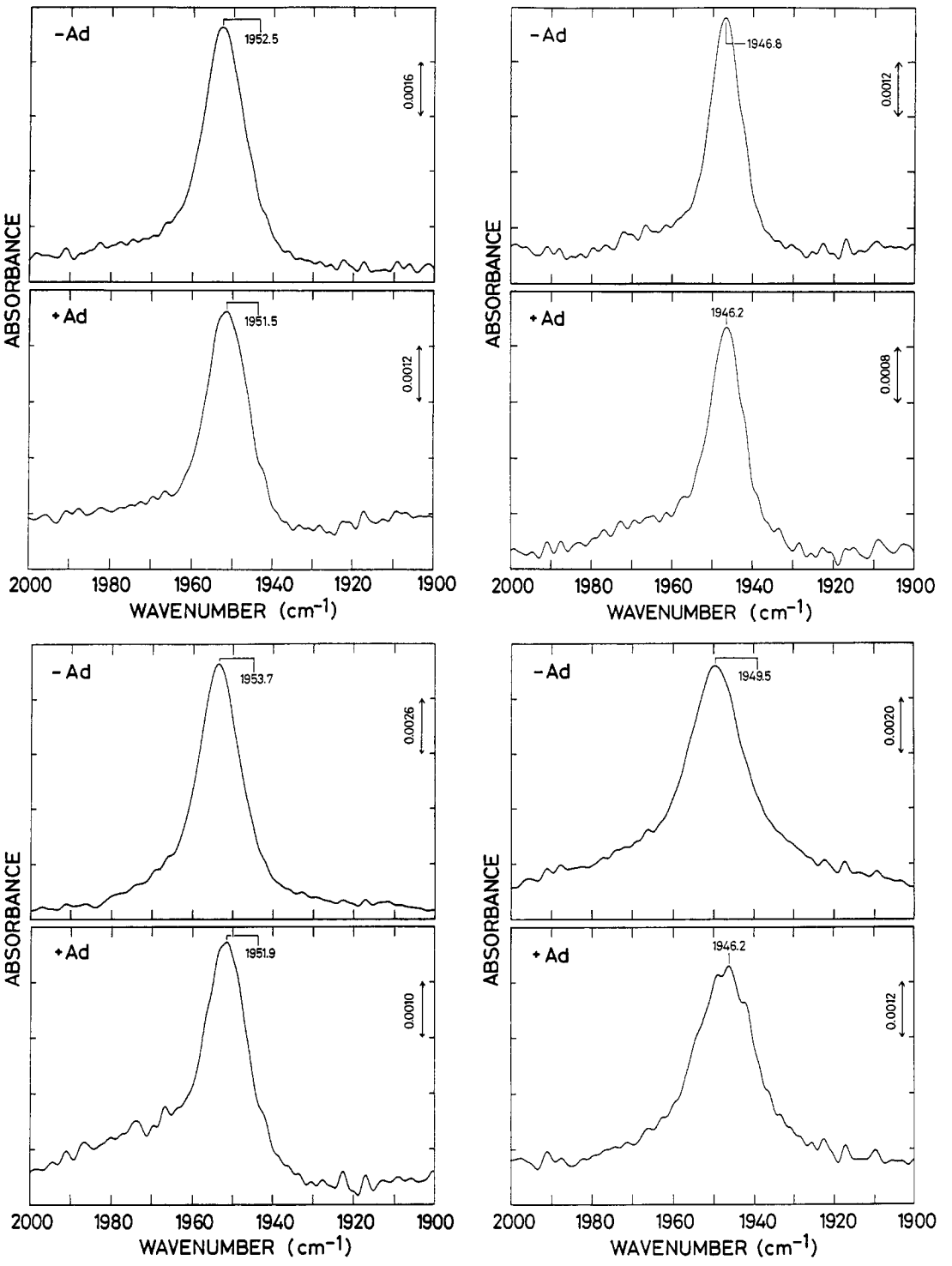

FIGURE 2: Effects of steroid side-chain group and reduced adrenodoxin binding to cytochrome P-450$_{scc}$ on the bound C–O stretching infrared vibration. (A, Top left) Substrate-free form; (B, bottom left) cholesterol-bound form; (C, top right) 22(*S*)-OH-cholesterol-bound form; (D, bottom right) 20(*S*)-OH-cholesterol bound form. Conditions: nominal spectral resolution, 2.0 cm^{-1}; spectral accumulation, 100 cycles (~40 min); temperature, 4 °C; light path length, 51 μm. Cytochrome P-450$_{scc}$–CO complex concentrations were 300–500 μM, and 20% molar excess of adrenodoxin was added for each C–O complex in 10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA.

in concert, whereas in the presence of (reduced) adrenodoxin the formation of the C–O band at 1951.4 cm^{-1} was much faster than that of the 1934.3 cm^{-1} C–O band. Appearance of the two C–O infrared bands was not due to the formation of the denatured form of cytochrome P-450$_{scc}$ since there was only a small formation of "P-420" judged with the visible absorption spectra of the same samples, and this P-420 may be responsible for a broad band centered around 1960 cm^{-1} beneath the strong 1952-cm^{-1} band, which could be revealed by the spectral deconvolution technique (see below). At the end of the reaction nearly all of the cytochrome P-450$_{scc}$ heme centers were reduced on the basis of the visible absorption spectroscopy and the C–O infrared band peak positions were found at 1951.9 and 1934.3 cm^{-1} in the absence of adrenodoxin and at 1952.2 and 1934.2 cm^{-1} in the presence of (reduced) adrenodoxin.

An additional important observation for the ferrous–CO cytochrome P-450$_{scc}$–22(*R*)-OH-cholesterol complex was the strong temperature dependence of the two C–O stretching band intensities (Figure 3). In the absence of adrenodoxin,

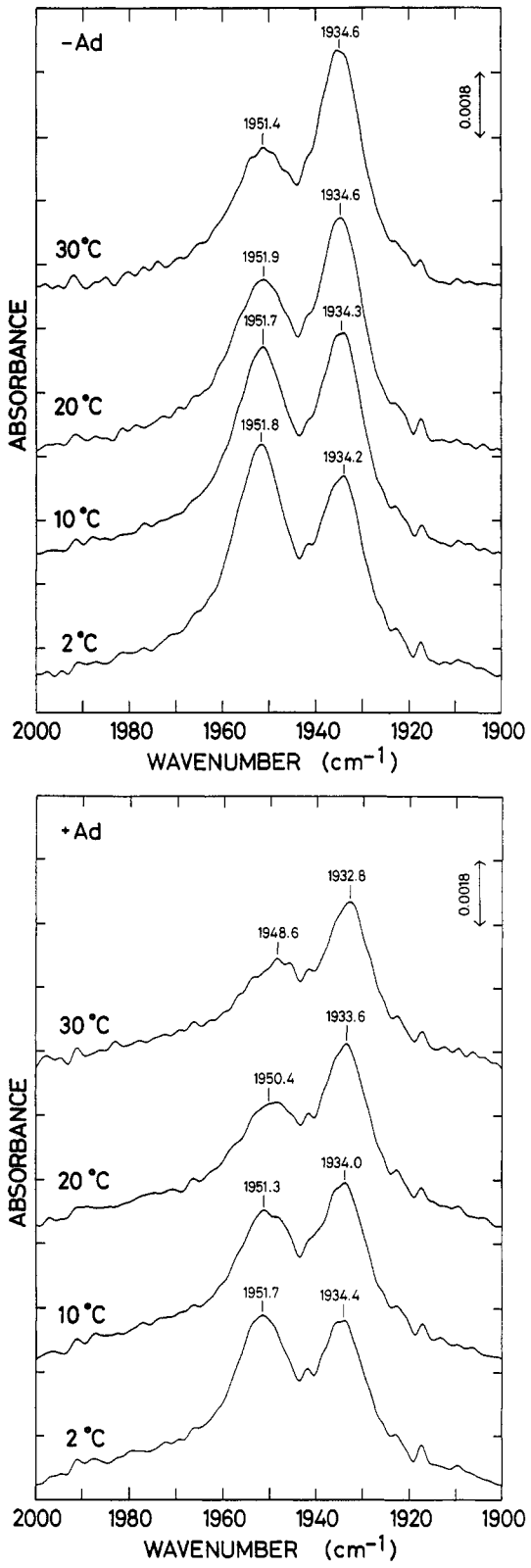

FIGURE 3: Temperature dependence of the bound C–O infrared band spectra of cytochrome P-450$_{scc}$–22(R)-OH-cholesterol complex in the absence (A, top) or in the presence (B, bottom) of reduced adrenodoxin. Conditions are the same as in Figure 2, except the temperature. Temperatures of the sample is indicated in the figure.

it seems that there is a temperature-dependent equilibrium between the two C–O infrared bands at 1951.9 and 1934.3 cm^{-1} (Figure 3A); at lower temperature (2 °C) both the 1951.9- and the 1934.3-cm^{-1} band are dominant but, at higher temperature (30 °C), the 1934.3-cm^{-1}-band intensity is much stronger than that of the 1951.9-cm^{-1} band. However, a careful

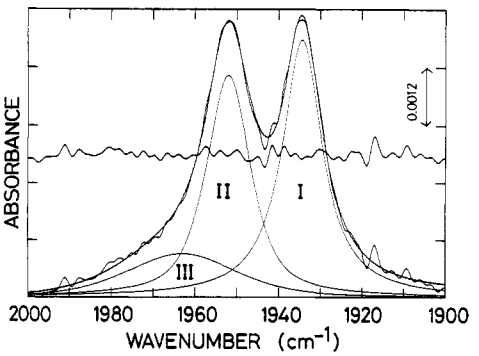

FIGURE 4: Deconvolution of the C–O infrared bands of cytochrome P-450$_{scc}$–22(R)-cholesterol-CO complex in the absence of adrenodoxin at 4 °C. Deconvolution procedure is described under Materials and Methods.

examination of the spectra suggests that the decrease of the 1951-cm^{-1}-band intensity is not parallel to the increase of the 1934-cm^{-1}-band intensity when the temperature is raised. In the presence of reduced adrenodoxin, such a temperature-dependent equilibrium does not exist; the formation of the 1952.2-cm^{-1} C–O band is suppressed at higher temperature (30 °C), whereas the 1934.2-cm^{-1} band is not influenced by temperature change (Figure 3B).

To clarify these points, the spectral deconvolutions of the C–O bands at various temperature (from 2 to 30 °C) were conducted. A typical deconvolution of the C–O infrared bands (at 4 °C) is shown in Figure 4. Three symmetric bands, namely, band III around 1960 cm^{-1} with $\Delta\nu_{1/2}$ = 31 cm^{-1} of 60% Gaussian/40% Lorentzian character, band II around 1952 cm^{-1} with $\Delta\nu_{1/2}$ = 12.6 cm^{-1} of 30% Gaussian/70% Lorentzian character, and band I around 1934 cm^{-1} with $\Delta\nu_{1/2}$ = 11.6 cm^{-1} of pure Lorentzian character, were required to obtain a reasonable fitting to the C–O bands, regardless of the temperature measured, in the absence of reduced adrenodoxin (Figure 4). Essentially similar results were obtained for the deconvolution analysis of the C–O bands in the presence of reduced adrenodoxin, except the $\Delta\nu_{1/2}$ = 12.6 cm^{-1} for band I (the 1934-cm^{-1} band). Temperature-dependent changes of each deconvoluted band area in the absence or in the presence of reduced adrenodoxin are shown in Figure 5. In the absence of reduced adrenodoxin, the decrease of the band II intensity with elevating temperature is compensated by the increase of the band I intensity (Figure 5, bottom). On the other hand, in the presence of reduced adrenodoxin, the sharp decrease of the band II intensity is only partly compensated by the increase of the band III intensity and the band I intensity remains at the same level upon the elevation of temperature (Figure 5, top). These results are fully consistent with the superficial interpretation of Figure 3. Therefore, the decrease of the population of the CO-bound form in the presence of reduced adrenodoxin should occur with elevating temperature. Indeed, the visible absorption spectra of the cytochrome P-450$_{scc}$–22(R)-OH-cholesterol–CO complex in the presence of reduced adrenodoxin at higher temperature indicated the increase of the absorbance around the 410-nm region compared to those measured at lower temperature, suggesting the increase of the five-coordinated reduced species (spectra not shown).

C–O Infrared Band of Cytochrome P-450$_{11\beta}$. In the absence of adrenodoxin the ferrous–CO complex of cytochrome P-450$_{11\beta}$ showed a very sharp C–O stretching infrared absorption at 1937.3 cm^{-1} with $\Delta\nu_{1/2}$ = 8.8 cm^{-1}. Addition of (reduced) adrenodoxin did not show any significant change on this band (spectra not shown) (see Table I). It must be mentioned that

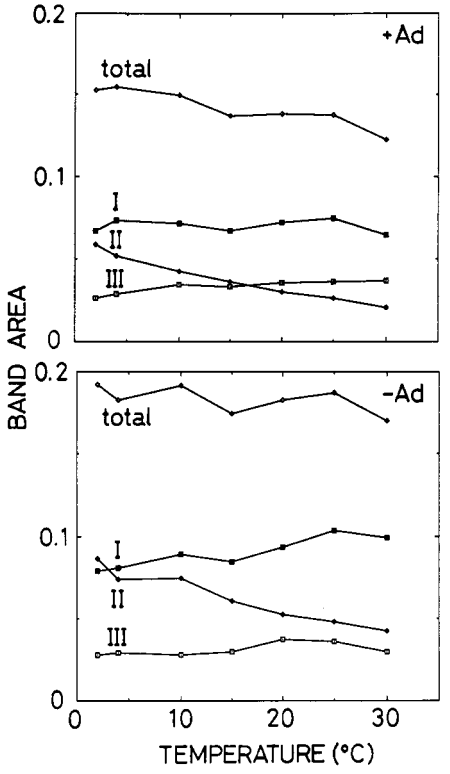

FIGURE 5: Temperature dependence of the band area of the deconvoluted C–O bands in the absence (top) or in the presence (bottom) of reduced adrenodoxin. The ordinates are in arbitrary units.

the cytochrome P-450$_{11\beta}$ samples used were in the substrate [deoxycorticosterone (DOC)]-complexed state.

DISCUSSION

Comparison of the C–O Stretching Band of Cytochrome P-450$_{scc}$ with That of Cytochrome P-450$_{cam}$. The C–O stretching band peak of cytochrome P-450$_{scc}$ in the substrate-free form was observed at 1952.5 cm^{-1} as a relatively sharp and symmetric band shape ($\Delta\nu_{1/2}$ = 12.6 cm^{-1}). This relatively narrow bandwidth of the C–O stretching band in the substrate-free form is a marked contrast to that of the corresponding band of cytochrome P-450$_{cam}$. O'Keefe et al. (1978) reported that two C–O stretching bands at 1963 (main) and 1942 cm^{-1} with half-bandwidths of 11–12 and 19–21 cm^{-1}, respectively, overlapped in the IR spectra of cytochrome P-450$_{cam}$ in the substrate (camphor)-free form. Later, Jung and Marlow (1987) reported that the very broad C–O infrared band of cytochrome P-450$_{cam}$ in the substrate-free form was composed of at least six bands with the main peak at 1949 cm^{-1} at room temperature (25 °C). The cause of this discrepancy is probably due to the extent of the contribution from the denatured P-420 form [the band peak at 1966 cm^{-1} was reported by O'Keefe et al. (1978)]. Both the existence of many C–O stretching modes indicative of the presence of "conformational substates" at the active site (heme pocket) (Frauenfelder et al., 1988; Jung & Marlow, 1987) and the vulnerability being converted easily to the P-420 form for the substrate-free form of cytochrome P-450$_{cam}$ suggest that, at least for the substrate-free CO-reduced state, the active site of cytochrome P-450$_{cam}$ has a more flexible structure than that of cytochrome P-450$_{scc}$. The conformational multiplicity (or flexibility) of cytochrome P-450$_{cam}$ at the active site seems lost upon substrate (camphor) binding; only one relatively sharp and symmetric band is reported to appear at 1940 cm^{-1} with $\Delta\nu_{1/2}$ = 13 cm^{-1} (O'Keefe et al., 1978; Jung & Marlow, 1987). The restriction of the conformational substates at the active site upon substrate binding may be understood as due to the steric hindrance of C–O binding(s) by the bound substrate molecule and/or due to the decrease of the flexibility of the active site by the formation of additional contacts with the different part of the active site mediated by the substrate molecule.

Conformational Abnormality of Cytochrome P-450$_{scc}$–20(S)-Hydroxycholesterol Complex. The extremely broadened C–O infrared bands of the 20(*S*)-OH-cholesterol complexes ($\Delta\nu_{1/2}$ = 17–18 cm^{-1}) compared to those of other steroids complexes ($\Delta\nu_{1/2}$ ranges from 10 to 13 cm^{-1}) are very interesting (Figure 2D and Table I). Physiologically, 20(*S*)-OH-cholesterol is not a proper intermediate, although the side-chain cleavage reaction still occurs with 20(*S*)-OH-cholesterol as substrate but at a reduced rate compared with 22(*R*)-OH-cholesterol, the first physiological intermediate (Morisaki et al., 1976). The abnormal nature of the heme–ligand moiety of the ferrous cytochrome P-450$_{scc}$–20(*S*)-OH-cholesterol complex was indeed revealed clearly by EPR spectroscopy using nitric oxide (NO) as an EPR probe (Tsubaki et al., 1987a). In that study it was shown that, upon addition of substrates other than 20(*S*)-OH-cholesterol, the EPR spectra exhibited signals having rhombic symmetry, whereas upon addition of 20(*S*)-OH-cholesterol the EPR spectra showed striking changes exhibiting signals with less rhombic symmetry. The EPR signals with less rhombic symmetry were considered to indicate polar surroundings of the ferrous heme–NO center of the cytochrome P-450$_{scc}$–20(*S*)-OH-cholesterol complex due to its peculiar conformational flexibility (Tsubaki et al., 1987a). The broadened C–O stretching infrared bands of cytochrome P-450$_{scc}$–20(*S*)-OH-cholesterol complexes observed are fully consistent with this view. If the vibrating C–O dipole interacts with its environment (water solvents in the heme cavity) to have a wide range of energies, the band becomes broadened, reflecting the energy distribution. Thus, the wider C–O stretching infrared band of cytochrome P-450$_{scc}$–20(*S*)-cholesterol complex compared to those of other cytochrome P-450$_{scc}$–steroid complexes indicates the stronger interaction of the ferrous–CO moiety with polar solvents in the active site. Indeed, our recent EPR study on the photoinduced intermediates of nitric oxide complexes of ferric cytochrome P-450$_{scc}$–steroid complexes confirmed this view (Hori et al., 1992).

Interactions of Steroid Side-Chain Group with Heme-Bound Carbon Monoxide. The steroid binding specificity of cytochrome P-450$_{scc}$ must be determined by specific interactions of these side-chain groups with amino acid residues at the substrate-binding site. Among the steroids used, 22(*R*)-OH-cholesterol and 20(*R*),22(*R*)-dihydroxycholesterol are of particular interest not only because these are physiological intermediates of the cholesterol side-chain cleavage reaction but because the direct interactions between these side-chain hydroxyls with the heme-bound ligands can be monitored by various techniques. Tuckey and Kamin (1983) reported that the dissociation constant (K_d) for CO, determined by titration of the reduced cytochrome P-450$_{scc}$ with CO, increased from 0.3 μM for the substrate-free state to 0.7, 5.3, 100, and 213 μM, respectively, for cholesterol-, 20(*S*)-OH-cholesterol-, 22(*R*)-OH-cholesterol-, and 20(*R*),22(*R*)-dihydroxycholesterol-complexed states. The dramatic increases of the dissociation constant (K_d) in 22(*R*)-OH-cholesterol- and 20(*R*),22(*R*)-dihydroxycholesterol-complexed states are reported to be mainly due to the decrease of the association rate constant (k_{on}) for CO binding (Tuckey & Kamin, 1983). It is further proposed on the basis of the specific interference

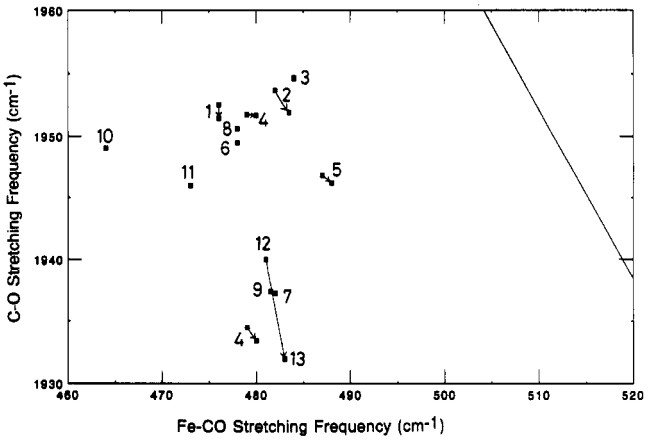

FIGURE 6: Effects of substrate and reduced adrenodoxin bindings to cytochrome P-450$_{scc}$ and cytochrome P-450$_{11\beta}$ on the vibrational modes (ν_{Fe-CO}, ν_{C-O}) of heme-bound C–O. Effects of reduced adrenodoxin (or putidaredoxin) binding are indicated by arrows. Corresponding data for cytochrome P-450$_{cam}$ from Makino et al. (1984) are also included for comparison. The straight line in the upper right corner is for various Fe^{2+}–CO–porphyrin–imidazole systems obtained by Li and Spiro (1988). Cytochrome P-450$_{scc}$ (1–8): 1, substrate free; 2., cholesterol; 3, 25-OH-cholesterol; 4, 22(*R*)-OH-cholesterol; 5, 22(*S*)-OH-cholesterol; 6, 20(*S*)-OH-cholesterol; 7, 20(*R*),22(*R*)-dihydroxycholesterol; 8, 22-ketocholesterol. Cytochrome P-450$_{11\beta}$ (9): 9, deoxycorticosterone. Cytochrome P-450$_{cam}$ (10–13): 10, substrate free; 11, norcamphor; 12, camphor; 13, camphor plus putidaredoxin.

of binding of 22(*R*)-OH-cholesterol by heme-bound carbon monoxide (but not oxygen) that the 22(*R*)-OH group localizes on a line perpendicular to the heme plane, between 2 and 3 Å from the iron (Heyl et al., 1986). The direct observation of such a specific interference of CO binding to ferrous heme iron by 22(*R*)-hydroxy steroids was reported previously using resonance Raman spectroscopy (Tsubaki et al., 1987), and this was confirmed in the present study.

The evaluation of the interaction between the steroid side-chain group and the heme-bound CO for cytochrome P-450–CO complexes was conducted on the ν_{Fe-CO} vs ν_{C-O} plot (Tsubaki et al., 1985). As shown in Figure 6, we could not see any negative correlation between ν_{Fe-CO} and ν_{C-O} for these cytochrome P-450$_{scc}$–CO complexes in contrast to the Fe^{2+}–porphyrin–CO–complexes having imidazole ligands, which showed strong negative correlation (Tsubaki & Ichikawa, 1985; Tsubaki et al., 1986; Kerr & Yu, 1988; Li & Spiro, 1988). Rather, inspection of the ν_{Fe-CO} vs ν_{C-O} plot for these cytochrome P-450–CO complexes revealed the existence of two groups of CO binding to the heme (Figure 6). All of the species showing the C–O stretching band in the region from 1930 to 1940 cm^{-1} are the complexes with substrates exhibiting higher turnover numbers [i.e., 22(*R*)-OH-cholesterol- and 20(*R*),22(*R*)-dihydroxycholesterol–cytochrome P-450$_{scc}$ complexes, cytochrome P-450$_{11\beta}$–deoxycorticosterone complex, and cytochrome P-450$_{cam}$–camphor complex in the presence or absence of putidaredoxin], whereas those showing the C–O band peak in the region above 1945 cm^{-1} are from cytochromes P-450 without substrate or cytochromes P-450 complexed with poorly catalyzed substrates [i.e., cholesterol, 25-OH-cholesterol, 22(*S*)-OH-cholesterol, 20(*S*)-OH-cholesterol, 22-ketocholesterol] (Morisaki et al., 1976; Iwahashi et al., 1991; White et al., 1984; Atkins & Sligar, 1987; Sato et al., 1978). Thus, as a working hypothesis we propose that the substrate binding configurations giving the C–O infrared vibration in the region from 1930 to 1940 cm^{-1} represent "productive substrate bindings", whereas the ones showing the C–O stretching vibrations in the region above 1945 cm^{-1} indicate "nonproductive substrate bindings". It must be mentioned, howevered, that "nonproductive" is not intended to mean the absence of metabolism but is implying the "inefficient" enzymatic reaction.

The two C–O infrared bands for the cytochrome P-450$_{scc}$–22(*R*)-OH-cholesterol complex indicate the coexistence of two different (local) conformations (or "conformational substates"; Frauenfelder et al., 1988) at the heme–CO site (the active center) in the temperature-dependent equilibrium. The appearance of these two conformational substates at the heme–CO site is most likely to be imposed by direct (steric) interaction between the heme-bound CO and the side-chain group of 22(*R*)-OH-cholesterol. Although the stereochemical mechanism of the temperature-dependent equilibrium between band II (1951 cm^{-1}) and band I (1934 cm^{-1}) in the absence of adrenodoxin is not clear, the preference of the band I (1934 cm^{-1}) species at higher temperature (>30 °C) is a physiologically reasonable mechanism for the efficient enzymatic reaction. This observation seems consistent with our working hypothesis.

The negative correlation between ν_{Fe-CO} and ν_{C-O} for a large number of CO adducts of Fe^{2+}–porphyrins having imidazole ligands is considered to reflect the role in Fe–CO bonding of the back-donation of iron electrons in the filled dπ orbitals to the empty π^* orbitals of the CO ligand. As back-donation increases, the Fe–CO bond becomes stronger, whereas the C–O bond strength decreases, resulting in the frequency change of two vibrations in an inverse direction (Li & Spiro, 1988). The deviation (positive or negative) of the plots from this straight line can occur when the fifth axial ligand is a weaker or stronger σ-donor than imidazole, as shown in the cytochrome P-450 family (the fifth ligand is a strongly donating thiolate ligand) (Tsubaki & Ichikawa, 1985). However, as long as the strength of the thiolate ligand is the same, a negative correlation between ν_{Fe-CO} and ν_{C-O} among the cytochrome P-450 family should still be expected. Recently, Park et al. (1991) and Augspurger et al. (1991) proposed that a weak electric field originating from the distal residue (such as His), together with a more global field term arising from the total charge distribution in a protein molecule, modifies the charge distribution in bound CO, which then modulates the degree of back-bonding from the iron and, therefore, determines the C–O stretching frequencies, on the basis of ^{17}O and ^{13}C NMR chemical shift measurements for various carbonyl hemoproteins. However, their view cannot explain satisfactorily the absence of the negative correlation since there is no distal His residue (or other polar residues close enough to the bound CO) in the cytochrome P-450 family (Poulos et al., 1985, 1987). Thus, our present observations on cytochrome P-450$_{scc}$ indicate that other possible controlling factors, such as direct steric hindrance from substrate and water solvent at the active site depending on the substrate bound, cannot be neglected. The polarity primarily derived from water solvents at the active site determines the C–O infrared bandwidth, whereas the steric hindrance caused by bound substrate may influence directly the C–O bond strength without affecting considerably the Fe–CO bond by an unknown mechanism(s).

Modulation of the Interactions between Steroid Side-Chain Group and Heme-Bound Carbon Monoxide by Adrenodoxin Binding. We have proposed that adrenodoxin may have another positive role in the cholesterol side-chain cleavage reaction, besides the transfer of an electron to the heme of cytochrome P-450$_{scc}$, on the basis of EPR studies (at 77 K) on ferrous–NO complexes of cytochrome P-450$_{scc}$ (Tsubaki et al., 1988). In the present study strong evidence to indicate

the role of adrenodoxin on the modulation of substrate binding configuration at the substrate-binding site has emerged. Apparently reduced adrenodoxin binding to the cytochrome P-450$_{scc}$–22(*R*)-OH-cholesterol complex causes a change in the interaction of steroid side-chain with heme-bound CO in a direction to favor the productive substrate binding configuration, i.e., the formation of band I (1934 cm^{-1}), by the interference of the nonproductive substrate binding, particularly at higher temperature (above 30 °C). In this case, however, the C–O band intensity of band I (1934 cm^{-1}) did not increase at all when the band II (1951 cm^{-1}) band intensity decreased with rising temperature.

Influences of the reduced adrenodoxin binding on the frequencies of heme-bound carbon monoxide for other cytochrome P-450$_{scc}$–steroid complexes were small but distinct and have a clear tendency. The C–O stretching frequency decreases by 0.1–3.3 cm^{-1}, whereas the Fe–CO stretching frequency increases by 0–1.5 cm^{-1} upon binding of (reduced) adrenodoxin (Table I). Similar influences on the C–O and Fe–CO stretching frequencies have been reported for putidaredoxin binding to cytochrome P-450$_{cam}$–CO complex in the camphor-bound form (Makino et al., 1984). The extent of the frequency shifts was much larger for cytochrome P-450$_{cam}$; the Fe–CO stretching frequency shifted by 2 cm^{-1} (from 481 to 483 cm^{-1}), and the C–O stretching frequency shifted by as much as 8 cm^{-1} (from 1940 to 1932 cm^{-1}). The tendency of the frequency shifts upon adrenodoxin (or putidaredoxin) binding can be visualized more clearly in Figure 6. The larger effect of the putidaredoxin binding than of the adrenodoxin binding on the C–O frequency must be a manifestation of the greater conformational flexibility of the cytochrome P-450$_{cam}$ active site than that of cytochrome P-450$_{scc}$ as noted in the previous section.

We have recently proposed the adrenodoxin binding site of cytochrome P-450$_{scc}$ by a specific chemical modification technique (Tsubaki et al., 1989a). This site is most likely to be formed with K and L helices [by cytochrome P-450$_{cam}$ notation; see Poulos et al. (1985, 1987)] at the proximal side of the heme iron. The positively charged residues (Lys and Arg) at this site may have important roles in the interaction with negatively charged adrenodoxin (Lambeth et al., 1984). There is no report, as far as we know, about the direct identification of the putidaredoxin binding site on cytochrome P-450$_{cam}$, but we believe that putidaredoxin binds to cytochrome P-450$_{cam}$ in a fashion similar to that of adrenodoxin. Indeed, an indirect method indicates that putidaredoxin may bind on the proximal surface of nearest approach to the cytochrome P-450$_{cam}$ heme prosthetic group (Stayton et al., 1989).

The substrate (steroid) binding site of cytochrome P-450$_{scc}$ should locate at the distal side of a heme prosthetic group like a cytochrome P-450$_{cam}$–camphor complex as revealed by X-ray crystallographic studies (Poulos et al., 1985, 1987). Thus, the binding of adrenodoxin on the proximal surface of cytochrome P-450$_{scc}$ must somehow propagate its binding to the distal side of the heme prosthetic group to modulate the interaction of the steroid side chain and the heme-bound ligand. Such propagation is most likely via protein moiety (polypeptide bond) rather than the heme prosthetic group. Therefore, cytochrome P-450$_{scc}$ is still flexible enough to transmit the effect of the adrenodoxin binding to the substrate binding site. In this context our recent study on the interaction of reduced adrenodoxin with ferrous cytochrome P-450$_{scc}$–phenyl isocyanide complex (Tsubaki et al., 1989b) has a very important meaning. The interaction of reduced adrenodoxin with ferrous cytochrome P-450$_{scc}$–phenyl isocyanide complex was modulated considerably by temperature in the region from 10 to 30 °C, the same region as in the present case for cytochrome P-450$_{scc}$–22(*R*)-OH-cholesterol complexes in the presence or absence of reduced adrenodoxin. These observations must be considered as an expression of the conformational flexibility of cytochrome P-450$_{scc}$ in the ambient temperature.

In conclusion, we emphasize the importance of the conformational flexibility of cytochrome P-450$_{scc}$ for the enzymatic reaction to proceed not only because such flexibility is essential to the substrate bindings to the active site (otherwise substrate cannot approach the active site located inside the protein matrix) but also because such flexibility may have important roles for the electron-transfer reaction from reduced adrenodoxin to the active site (Tsubaki et al., 1989a) and for the oxygen activation reaction followed by the region-specific (and efficient) hydroxylation reaction at this site. The absence of the negative correlation between the ν_{Fe-CO} and ν_{C-O} in cytochrome P-450$_{scc}$–CO complexes also may be related to this conformational flexibility.

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