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Inhibition of Electron Transfer from Adrenodoxin to Cytochrome P-450$_{scc}$ by Chemical Modification with Pyridoxal 5′-Phosphate: Identification of Adrenodoxin-Binding Site of Cytochrome P-450$_{scc}$†

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ABSTRACT: Covalent modification of cytochrome P-450$_{scc}$ (purified from bovine adrenocortical mitochondria) with pyridoxal 5′-phosphate (PLP) was found to cause inhibition of the electron-accepting ability of this enzyme from its physiological electron donor, adrenodoxin, without conversion to the "P-420" form. Reaction conditions leading to the modification level of 0.82 and 2.85 PLP–Lys residues per cytochrome P-450$_{scc}$ molecule resulted in 60% and 98% inhibition, respectively, of electron-transfer rate from adrenodoxin to cytochrome P-450$_{scc}$ (with β-NADPH as an electron donor via NADPH–adrenodoxin reductase and with phenyl isocyanide as the exogenous heme ligand of the cytochrome). It was found that covalent PLP modification caused a drastic decrease of cholesterol side-chain cleavage activity when the cholesterol side-chain cleavage enzyme system was reconstituted with native (or PLP-modified) cytochrome P-450$_{scc}$, adrenodoxin, and NADPH–adrenodoxin reductase. Approximately 60% of the original enzymatic activity of cytochrome P-450$_{scc}$ was protected against inactivation by covalent PLP modification when 20% mole excess adrenodoxin was included during incubation with PLP. Binding affinity of substrate (cholesterol) to cytochrome P-450$_{scc}$ was found to be increased slightly upon covalent modification with PLP by analyzing a substrate-induced spectral change. The interaction of adrenodoxin with cytochrome P-450$_{scc}$ in the absence of substrate (cholesterol) was analyzed by difference absorption spectroscopy with a four-cuvette assembly, and the apparent dissociation constant (K_s) for adrenodoxin binding was found to be increased from 0.38 μM (native) to 33 μM (covalently PLP modified). These results indicate that the binding of only a few PLP molecules to a Lys residue(s) that is (are) essential to the interaction with adrenodoxin on the surface of cytochrome P-450$_{scc}$ caused interference of the electron transfer from adrenodoxin, leading to a drastic decrease of the cholesterol side-chain cleavage activity. Distribution of fluorescence due to the PLP–Lys residues in tryptic peptides of cytochrome P-450$_{scc}$(SF) that had been previously treated with PLP and $NaBH_4$ in the presence or absence of adrenodoxin was analyzed by reverse-phase HPLC. Only two peptides were specifically labeled with PLP, and this specific modification was prevented by the presence of the protector, adrenodoxin. These two peptides were purified, and their primary structures were determined. The sequences of these two peptides overlapped (one peptide from residue 378 to residue 385, the other from residue 369 to residue 381 of the protein), and either one of adjacent Lys residues (Lys 381 or Lys 377) is specifically modified with PLP in each peptide. The region containing this putative adrenodoxin-binding site is highly homologous to the corresponding region of bovine mitochondrial cytochrome P-450$_{11\beta}$.

Adrenal mitochondria contain two enzymes that catalyze the transfer of reducing equivalents from β-NADPH to cytochrome P-450$_{scc}$—a low molecular weight iron–sulfur protein (adrenodoxin) and a β-NADPH-specific flavoprotein (adrenodoxin reductase) containing a single FAD (Jefcoate, 1986; Lambeth et al., 1982). Adrenodoxin contains an active center consisting of two iron atoms, each coordinated tetrahedrally by two cysteinyl sulfurs and two acid-labile sulfur atoms. Although in the oxidized state both irons are in the +3 state, only a single electron can be introduced into a Fe_2S_2 core from adrenodoxin reductase (Orme-Johnson & Beinert, 1969; Estabrook et al., 1973).

To elucidate the molecular mechanism for the electron transport to cytochrome P-450$_{scc}$, a hypothesis, "shuttling of adrenodoxin between adrenodoxin reductase and cytochrome P-450$_{scc}$", has been proposed (Lambeth et al., 1979, 1982). During a catalytic cycle of electron transfer, according to this hypothesis, adrenodoxin forms initially a 1:1 complex with adrenodoxin reductase. Reduction of the iron–sulfur center promotes dissociation of this complex and allows binding and subsequent electron transfer to the cytochrome P-450. The strength of interaction for both proteins is reduced considerably at high ionic strength, indicating the contribution of electro-

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static interactions to the binding (Lambeth et al., 1979; Hanukoglu et al., 1981b). Since the binding of adrenodoxin to adrenodoxin reductase appears to be competitive with its binding to cytochrome P-450$_{scc}$ (Seybert et al., 1978, 1979; Hanukoglu & Jefcoate, 1980), it has been proposed that the same site (or overlapping sites) on adrenodoxin is (are) involved in the interaction both with adrenodoxin reductase and with cytochrome P-450$_{scc}$ (Lambeth et al., 1982). This proposal is in accordance with the shuttle mechanism.

Adrenodoxin is an unusually acidic protein (11 Asp and 7 Glu residues per 114 amino acid residues total)[1] (Tanaka et al., 1973; Hiwatashi et al., 1986; Bhasker et al., 1987) with an isoelectric point of 4.0 (Maruya et al., 1983). A water-soluble carbodiimide, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide, has been shown to modify carboxyl groups only in highly negatively charged environments, thus leading to a selective modification of a limited number of Asp and Glu residues. In adrenodoxin, only three residues are modified (Glu 74, Asp 79, and Asp 86) (Geren et al., 1984). This modification of adrenodoxin leads to diminished binding both to adrenodoxin reductase and to cytochrome P-450$_{scc}$ (Lambeth et al., 1984). Thus, these negatively charged residues on adrenodoxin presumably participate in interactions with positively charged residues (Lys and Arg residues) on the other proteins.

In the present study, we have used pyridoxal 5′-phosphate (PLP)[2] to clarify the interaction between cytochrome P-450$_{scc}$ and adrenodoxin. This reagent has been shown to modify amino groups (ϵ-NH_2 group of Lys residues or an α-NH_2 group) by forming a Schiff base; reduction of the Schiff base with $NaBH_4$ results in irreversible attachment of PLP to the amino groups (Benesch et al., 1972, 1982; Aviram & Schejter, 1973; Atanasov et al., 1984; Poulose & Kolattukudy, 1980). Hamamoto and Ichikawa (1984) showed that treatment of adrenodoxin reductase with PLP followed by the $NaBH_4$ reduction caused a selective modification of several Lys residues. The modification of an essential Lys residue(s) led to diminished binding of adrenodoxin to adrenodoxin reductase on the basis of a cytochrome *c* reductase assay. Motivated by this observation, we have extended our studies to the modification of amino groups of cytochrome P-450$_{scc}$ with PLP to see its effect on the interaction with adrenodoxin.

Experimental Procedures

Materials. $Na_2S_2O_4$, *N*-(trimethylsilyl)imidazole, pregnenolone, cholesterol, *N*,*N*-dimethylformamide, diethyl ether, NaCl, pyridoxal 5′-phosphate (PLP), $NaBH_4$, $MgCl_2$, EDTA, sodium dodecyl sulfate (SDS), NH_4HCO_3, acetonitrile, trifluoroacetic acid, and lysine were obtained from Wako Pure Chemical Industries, Inc. (Osaka). β-NADPH was from Kohjin Co., Ltd. (Tokyo). Emulgen 913 and Tween 20 were from Kao-Atlas. Sodium cholate and TPCK-treated trypsin (from bovine pancreas) were from Sigma (St. Louis, MO). Phenyl isocyanide was synthesized as previously described (Schmidt & Stern, 1929). [17,21,21,21-$^2H_4$]Pregnenolone was synthesized according to the method of Morisaki et al. (1985). Adrenodoxin, adrenodoxin reductase, and cytochrome P-450$_{scc}$ were purified from bovine adrenocortical mitochondria as described previously (Hiwatashi et al., 1976, 1986; Tsubaki et al., 1986a). Spinach ferredoxin was kindly donated by Dr. Masateru Shin of Kobe University, Kobe, Japan. Homogeneity was assessed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970).

Endogenous cholesterol and its hydroxylated intermediates, if any, bound to cytochrome P-450$_{scc}$ were removed during the purification by the continuous use of a nonionic detergent, Emulgen 913, in the buffer system (Tsubaki et al., 1986b). Emulgen 913 was then removed by adrenodoxin–Sepharose 4B column chromatography as previously described (Tsubaki et al., 1986a), yielding cytochrome P-450$_{scc}$ in substrate-free form. We term this form hereafter cytochrome P-450$_{scc}$(SF). The heme content of the purified cytochrome P-450$_{scc}$(SF) was 18–19 nmol/mg of protein. The cholesterol-bound form [cytochrome P-450$_{scc}$(Chol)] was prepared as previously described (Tsubaki et al., 1986a).

Modification of Cytochrome P-450$_{scc}$ with PLP. Modification of Lys residues of cytochrome P-450$_{scc}$(SF) was carried out at 25 °C in 20 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 0.1 mM EDTA, and 2.0 mM PLP (unless otherwise indicated) at a protein concentration of 200 μM ($\sim$10 mg/mL). Light was avoided during the reaction. At intervals, aliquots of the reaction mixture were removed and assayed immediately for electron-accepting activity from β-NADPH via adrenodoxin reductase and adrenodoxin (see below); the initial activity was measured before the addition of PLP. Irreversible modification was attained by reduction of the Schiff base by addition of solid $NaBH_4$ (final concentration of $NaBH_4$, 20 mM), and pH was monitored; usually there was no need to readjust the pH. This treatment also destroys the excess reagent. The reaction mixture was kept at room temperature for an additional 10 min and, then, was dialyzed extensively at 4 °C in the dark against 20 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. For control, the enzyme was treated similarly but without PLP. For protection experiments, a protecting agent [20% mole excess adrenodoxin, bovine serum albumin, or spinach ferredoxin relative to cytochrome P-450$_{scc}$(SF)] was added to the enzyme before the addition of PLP. Aliquots of the dialyzed sample were used for determinations of electron-accepting activity from β-NADPH, cholesterol side-chain cleavage activity, and spectral characterization as described below.

Cytochrome P-450$_{scc}$(Chol) was treated with PLP in the same way as for cytochrome P-450$_{scc}$(SF) to see the effect of the presence of cholesterol at substrate-binding site during the modification.

Measurements of Electron-Accepting Activity of Cytochrome P-450$_{scc}$ from β-NADPH. The electron-accepting activity of native (or PLP-modified) cytochrome P-450$_{scc}$ from β-NADPH was measured by taking advantage of the formation of the cytochrome P-450$_{scc}$(Fe^{2+})–phenyl isocyanide complex,[3] which is stable under aerobic conditions (Tsubaki et al., unpublished results). The reaction mixture contained

[1] Recently, the existence of at least a large part of additional 14 amino acid COOH-terminal segment in the mature protein was proved by Hiwatashi et al. (1986) and Bhasker et al. (1987).

[2] Abbreviations: PLP, pyridoxal 5′-phosphate; cytochrome P-450$_{scc}$(SF), cytochrome P-450$_{scc}$ in substrate-free form; cytochrome P-450$_{scc}$(Chol), cytochrome P-450$_{scc}$ complexed with cholesterol; EDTA, ethylenediaminetetraacetic acid.

[3] The nature of the bonding between various isocyanides (alkyl isocyanides and aromatic isocyanides) with various hemoproteins was described in Mims et al. (1983) and Wood et al. (1987). Earlier works about the interaction between isocyanides and cytochrome P-450 were reported in Imai and Sato (1966), Ichikawa and Yamano (1968), and Griffin and Peterson (1971). To our knowledge, there is no reference describing the precise nature of the interaction between phenyl isocyanide and cytochrome P-450$_{scc}$, except for a preliminary resonance Raman study on the cytochrome P-450$_{scc}$–phenyl isocyanide complex (Tsubaki et al., 1987). Detailed study of the ferrous cytochrome P-450$_{scc}$–phenyl isocyanide complex will be reported elsewhere.

15 μL of 200 μM cytochrome P-450$_{scc}$, 7.5 μL of 1.0 μM adrenodoxin, 7.5 μL of 1.0 μM adrenodoxin reductase, and 1.5 mL of 20 mM potassium phosphate buffer (pH 7.4) containing 0.47 mM phenyl isocyanide, 20% glycerol, 0.1% (v/v) Emulgen 913, 0.25% (w/v) sodium cholate, and 0.1 mM EDTA. Temperature of the reaction mixture was maintained at 25 °C by a water-jacketed cell holder in a Shimadzu UV-240 spectrophotometer. The reaction was started by adding 10 μL of 10 mM β-NADPH, and the absorbance change at 455 nm due to the formation of the heme(Fe^{2+})–phenyl isocyanide complex was recorded and analyzed. Emulgen 913 and sodium cholate were added in the electron-accepting activity assay system to produce cytochrome P-450$_{scc}$(Fe^{3+})–phenyl isocyanide complex in advance; otherwise, cholesterol at the substrate-binding site inhibits the binding of phenyl isocyanide to the heme of the cytochrome.

To see the effect of protection of the electron-accepting activity with adrenodoxin against the covalent PLP modification, we also measured the electron-accepting activity of cytochrome P-450$_{scc}$ under the same condition as described above except for adrenodoxin and adrenodoxin reductase, which were added in excess (adrenodoxin was 20% mole excess relative to cytochrome P-450$_{scc}$ and the concentration of adrenodoxin reductase was increased 4-fold). These changes caused an immediate reduction of all heme iron of native cytochrome P-450$_{scc}$ upon addition of β-NADPH. Therefore, the ratio of the absorbance change at 455 nm of the PLP-modified enzyme upon reduction with β-NADPH to the absorbance change upon reduction with $Na_2S_2O_4$ was taken as the extent of loss of electron-accepting activity from β-NADPH of the PLP-modified cytochrome P-450$_{scc}$.

Measurements of Cholesterol Side-Chain Cleavage Activities. Cholesterol (100 μg) dissolved in 50 μL of *N,N*-dimethylformamide was incubated with 2.0 mL of enzyme mixture containing 0.5 μM native (or PLP-modified) cytochrome P-450$_{scc}$, 10 μM adrenodoxin, and 0.15 μM adrenodoxin reductase in 20 mM potassium phosphate buffer (pH 7.4) in the presence of 0.1% (v/v) Tween 20 (final concentration). Tween 20 was included in the cholesterol side-chain cleavage activity assay system since this detergent is the most effective activator of the activity among various detergents (Takikawa et al., 1978). The cholesterol side-chain cleavage reaction was started by adding 0.4 mL of an NADPH-generating system. The NADPH-generating system consisted of 0.15 mL of 0.1 M $MgCl_2$, 0.05 mL of 0.1 M DL-isocitrate, 0.15 mL of 20 mM β-NADPH, and 0.02 mL of isocitrate dehydrogenase ($NADP^+$) (3 IU/mL) in a final volume of 2.87 mL. The reaction was performed at 37 °C in the dark and, at intervals, 1.0 mL of the reaction mixture was withdrawn; the reaction was terminated by the addition of 5 mL of dichloromethane containing 2.0 μg of [17,21,21,21-$^2H_4$]pregnenolone as internal standard. After a period of shaking with a mixer, steroids were extracted into the dichloromethane layer. Extraction was carried out once more without internal standard. The dichloromethane extract was concentrated, and the solvent was evaporated to dryness. *N*-(Trimethylsilyl)-imidazole (30 μL) was added to the residue in a test tube to convert pregnenolone to the trimethylsilyl ether. The quantitative analyses by selected ion monitoring were performed by a Hitachi M-80 GC-MS spectrometer using the ions at m/z 298 and m/z 302 (corresponding to the molecular ion minus trimethylsilanol) according to the method of Morisaki et al. (1985).

Spectroscopic Experiments. Absorption spectra of cytochrome P-450$_{scc}$ were recorded in 20 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA at 25 °C with a Shimadzu UV-240 spectrophotometer (or as otherwise indicated).

For the cholesterol binding study, a varying amount of cholesterol dissolved n 10 μL of *N,N*-dimethylformamide was added to 3.0 mL of native (or PLP-modified) cytochrome P-450$_{scc}$(SF) (1.69 μM) in 20 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA, and the mixture was incubated in the dark at 4 °C overnight. Binding of cholesterol to cytochrome P-450$_{scc}$(SF) was then analyzed from the difference spectra by measuring absorbance changes at 417 nm (low spin) and 392 nm (high spin).

Interaction of adrenodoxin with native (or PLP-modified) cytochrome P-450$_{scc}$(SF) was investigated by titration with adrenodoxin using a four-cuvette assembly (Lambeth et al., 1980) and a Hitachi 557 spectrophotometer. For each addition, adrenodoxin was added to the sample cuvette containing cytochrome P-450$_{scc}$ and to the reference cuvette containing buffer. An equal volume of buffer was also added to the second reference cuvette containing cytochrome P-450$_{scc}$. To avoid the effect of a residual contamination by cholesterol, which causes an increase of adrenodoxin binding affinity (Lambeth et al., 1980; Hanukoglu et al., 1981a), the titration experiments were performed in the presence of detergents [buffer; 10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.1% Emulgen 913, and 0.25% sodium cholate].

Cytochrome P-450$_{scc}$ content was determined by using the extinction coefficient of 131.9 mM^{-1} cm^{-1} in the substrate-free oxidized state as previously described (Tsubaki et al., 1986b). Protein concentration was determined by the biuret method (Gornall et al., 1949).

Identification and Purification of the Adrenodoxin-Binding-Site Peptides of Cytochrome P-450$_{scc}$. Cytochrome P-450$_{scc}$(SF) samples treated with PLP (0.5 mM or 2.0 mM)/$NaBH_4$ in the presence and absence of adrenodoxin (20% mole excess), respectively, were dialyzed against 0.1 M NH_4HCO_3 (pH 8.0) overnight in the dark at 4 °C. The samples were digested with TPCK-treated trypsin. The trypsin solution [1.0 mg/mL in 0.1 M NH_4HCO_3 (pH 8.0)] was added first to the sample solution to make an enzyme:protein ratio of 1:100; 2 h later, the same amount of trypsin solution was added again. The digestion was continued for 18 h at 37 °C in the dark. The digested samples were lyophilized and saved at −80 °C in the dark until use.

The digested samples were dissolved in 70% (v/v) formic acid and were chromatographed on a reverse-phase SynChropak RP-P (C18) (4.1 × 250 mm) column by using a Hitachi 655A HPLC system with a linear gradient (0–100%) of acetonitrile concentration in the presence of 0.1% (v/v) trifluoroacetic acid. Peptides were detected by absorbance at 210 nm and simultaneously by fluorescence at 398 nm (with excitation at 298 nm). The elution profiles were compared with each other, and putative adrenodoxin-binding-site peptides were identified. The peptides were collected, lyophilized, and rechromatographed under the same condition.

The amino acid sequences of the purified peptides were determined by automated Edman degradation on a gas-phase protein sequencer (Applied Biosystems, Model 470A) and a HPLC 8100 system from Spectra Physics. Pyridoxal 5′-phosphate–ε-*N*-lysine was synthesized by mixing lysine and pyridoxal 5′-phosphate in 1:1 molar ratio at pH 9.0 followed by $NaBH_4$ reduction (Atanasov et al., 1984). The main component was fractioned from the mixture by preparative paper chromatography (Whatman 3) using pyridine/buta-

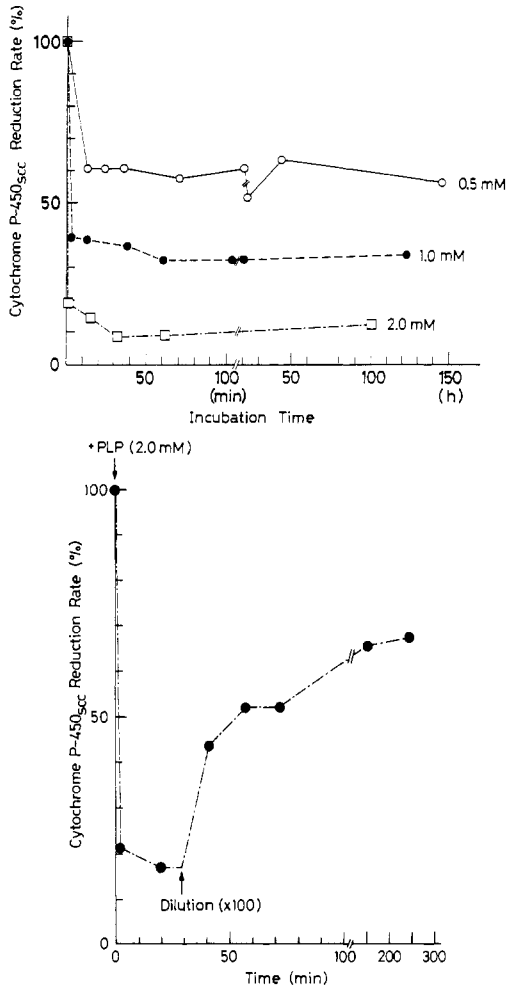

FIGURE 1: (Top) Time course of inhibition of electron-accepting activity of cytochrome P-450$_{scc}$ from β-NADPH by incubation of cytochrome P-450$_{scc}$(SF) with PLP. Cytochrome P-450$_{scc}$(SF) (200 μM) was incubated with indicated concentrations of PLP at 25 °C in the dark, and at intervals aliquots were withdrawn and assayed immediately for the electron-accepting activity. (Bottom) Restoration of the electron-accepting activity upon dilution of the PLP-incubated cytochrome P-450$_{scc}$(SF). The enzyme (200 μM) after incubation with 2.0 mM PLP at 25 °C in the dark for 30 min was diluted 100-fold with 20 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA and kept at 25 °C in the dark; at intervals aliquots were withdrawn and assayed immediately for the electron-accepting activity.

nol/acetic acid/water (2:3:1:4). The corresponding phenylthiohydantoin derivative was prepared on the gas-phase protein sequencer.

RESULTS

Inhibition of Electron-Accepting Activity by PLP Treatment. Incubation of cytochrome P-450$_{scc}$(SF) with PLP resulted in inhibition of electron-accepting activity from β-NADPH via NADPH–adrenodoxin reductase and adrenodoxin. The rate of inactivation of the electron-accepting activity by PLP and the extent of the inactivation with varying concentration of this inhibitor are shown in Figure 1 (top). The electron-accepting activity rapidly decreased in the first 10 min, and subsequently the rate of the inactivation was slower; after 30 min, there was little further change in the activity. The extent of the inactivation increased with increasing concentration of PLP. The inactivation of cytochrome P-450$_{scc}$(SF) electron-accepting activity by PLP was readily reversed by dilution [Figure 1 (bottom)] or dialysis. When the samples treated with varying concentration of PLP were

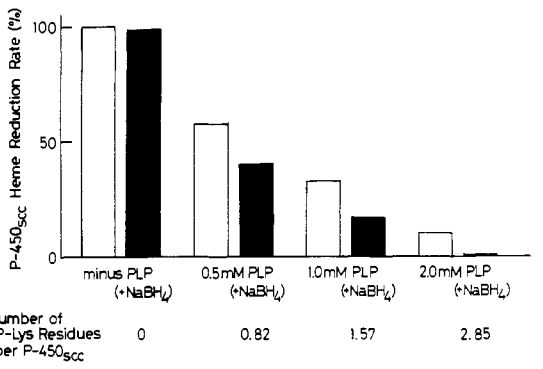

FIGURE 2: Effect of treatment with $NaBH_4$ on the electron-accepting activity of PLP-incubated cytochrome P-450$_{scc}$(SF) from β-NADPH. Cytochrome P-450$_{scc}$(SF) (200 μM) was incubated in the absence of PLP (minus PLP) or in the presence of indicated concentrations of PLP for 1 h at 25 °C in the dark, and an aliquot was withdrawn and assayed immediately for the electron-accepting activity (open bars). Irreversible (covalent) modification was attained by addition of solid $NaBH_4$ (final concentration, 20 mM); the sample was incubated for 10 min in the dark at 25 °C and its electron-accepting activity was measured (solid bars).

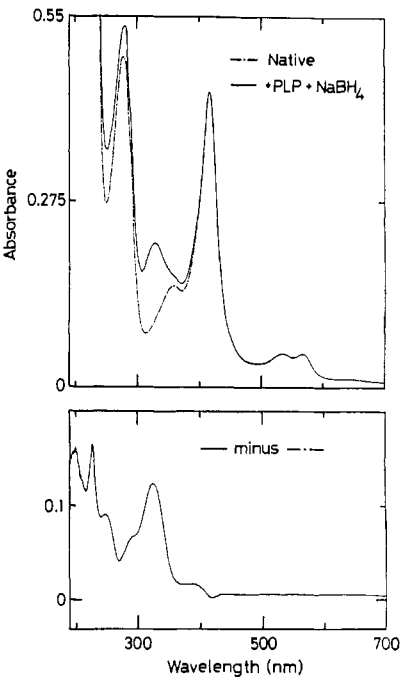

FIGURE 3: Effect of covalent PLP modification (incubation with PLP followed by $NaBH_4$ reduction) on an absorption spectrum of cytochrome P-450$_{scc}$(SF). (Upper) (—) Native cytochrome P-450$_{scc}$(SF); (- - -) (2.0 mM PLP + $NaBH_4$)-treated cytochrome P-450$_{scc}$(SF). (Lower) Difference spectrum; native minus (2.0 mM PLP + $NaBH_4$)-treated. Absorption spectra were recorded in 20 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA at 25 °C. Cytochrome P-450$_{scc}$(SF) concentration was 3.34 μM.

reduced with $NaBH_4$ prior to the electron-accepting activity assay, there were always further decreases of activity by about 15% (Figure 2). These observations suggest that PLP formed a Schiff base with an ϵ-amino group of Lys residues (or α-amino group). Thus, discrepancies in residual activities before and after the $NaBH_4$ treatment could be due partly to the dissociation of a noncovalent enzyme–PLP complex and mostly to the hydrolysis of the inactive Schiff base upon dilution to give active enzyme in the assay mixture (Poulose & Lolattukudy, 1980).

Effect of Covalent PLP Modification on Absorption Spectra of Cytochrome P-450$_{scc}$(SF). Upon reduction of PLP (2.0 mM) treated cytochrome P-450$_{scc}$(SF) with $NaBH_4$, a new

absorption band appeared at 325 nm (Figure 3), and when excited at 325 nm, this modified cytochrome P-450$_{scc}$ gave a fluorescence spectrum with a maximum at 395 nm (data not shown), characteristic of substituted pyridoxamine 5′-phosphate. There was no spectral perturbation after the PLP treatment followed by the $NaBH_4$ reduction in the absorption spectra of cytochrome P-450$_{scc}$(SF) in the oxidized state except for an appearance of the 325-nm band (Figure 3). In the CO-reduced state there was no indication of the formation of an inactive form P-420 upon this treatment (data not shown). There was no effect of the covalent PLP modification on visible absorption spectra of ferrous–phenyl isocyanide complexed state (chemically reduced with $Na_2S_2O_4$) and, also, there was no difference in phenyl isocyanide binding affinity between the native and the covalently PLP modified samples according to our spectroscopic titration experiments (data not shown). All these spectral indications suggested that the PLP treatment followed by $NaBH_4$ reduction did not alter the heme environment of cytochrome P-450$_{scc}$(SF).

The numbers of Lys residues modified by the PLP treatment at various concentrations followed by $NaBH_4$ reduction were estimated spectrophotometrically by using the extinction coefficient of pyridoxal 5′-phosphate–ϵ-*N*-lysine (9.72 mM^{-1} cm^{-1}) at 325 nm (Fischer et al., 1963) and are also indicated in Figure 2. It is believed that positively charged amino acid residues (Lys and Arg residues) on cytochrome P-450$_{scc}$ are responsible for the electrostatic interaction with negatively charged residues on adrenodoxin (Lambeth et al., 1984). Since the PLP treatment (at 2.0 mM) followed by $NaBH_4$ reduction caused the modification of only 3 of 33 Lys residues of the enzyme (Morohashi et al., 1984), it is very likely that an essential Lys residue(s) for the interaction with adrenodoxin is (are) selectively modified by this treatment, leading to the almost complete inhibition of the electron transfer from adrenodoxin to cytochrome P-450$_{scc}$. It is important to note that when only 0.82 Lys residue/P-450$_{scc}$ was modified with PLP (at 0.5 mM PLP), 60% of the original enzymatic activity was already abolished, suggesting that only a catalytically important Lys residue(s) was specifically modified with PLP under this condition.

Inhibition of Cholesterol Side-Chain Cleavage Activity by Covalent PLP Modification. Treatment of cytochrome P-450$_{scc}$(SF) with PLP followed by $NaBH_4$ reduction caused almost complete inhibition of the cholesterol side-chain cleavage reaction in a reconstituted enzyme assay system as shown in Figure 4 (top). Treatment with $NaBH_4$ alone did not cause any loss of the side-chain cleavage activity at all. Since the covalent PLP modification caused the loss of the electron-accepting ability of cytochrome P-450$_{scc}$(SF) from β-NADPH via NADPH–adrenodoxin reductase and adrenodoxin, it is reasonable to assume that the cholesterol side-chain cleavage reaction, which requires a total of six electrons for the completion of a reaction cycle (Shikita & Hall, 1974), was subsequently abolished.

Effect of Covalent PLP Modification of Cytochrome P-450$_{scc}$(SF) on Cholesterol Binding Affinity. There is a possibility that the loss of the cholesterol side-chain cleavage activity upon the covalent PLP modification may be due to the loss of substrate (cholesterol) binding ability in addition to the loss of the electron-accepting activity from β-NADPH. To clarify this point. we examined the effects of the covalent PLP modification on the cholesterol binding affinity of cytochrome P-450$_{scc}$. Binding of cholesterol to cytochrome P-450$_{scc}$ was measured by using substrate (cholesterol) induced optical absorption change from low- to high-spin state. A Line-

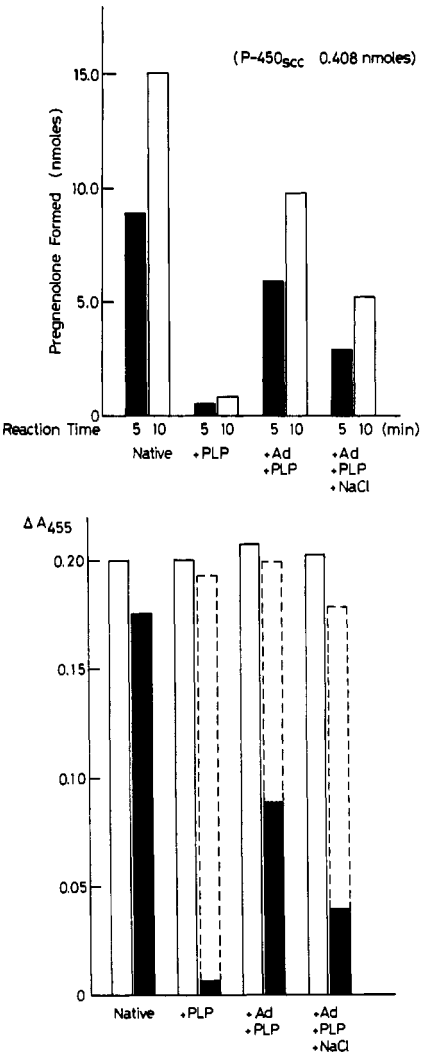

FIGURE 4: (Top) Inhibition of cholesterol side-chain cleavage activity by covalent PLP modification of cytochrome P-450$_{scc}$(SF) (+PLP), protection of activity by adrenodoxin (20% mole excess) against inactivation (+Ad + PLP), and inhibition of the protection of activity by adrenodoxin in the presence of 0.35 M NaCl during the PLP modification (+Ad + PLP + NaCl). Side-chain cleavage reaction time: (solid bars) 5 min; (open bars) 10 min. (Bottom) Inhibition of electron-accepting activity by the covalent PLP modification of cytochrome P-450$_{scc}$(SF) (+PLP), protection of activity by adrenodoxin against inactivation (+Ad + PLP), and inhibition of protection of activity by adrenodoxin in the presence of 0.35 M NaCl during the PLP modification (+Ad + PLP + NaCl). Absorbance change at 455 nm due to formation of heme(Fe^{2+})–phenyl isocyanide complex was monitored as described under Experimental Procedures. (Open bars) Chemically reduced with $Na_2S_2O_4$; (solid bars) enzymatically reduced with β-NADPH; (broken-line bars) enzymatically reduced and then chemically reduced with $Na_2S_2O_4$. Final cytochrome P-450$_{scc}$(SF) concentration was 2.57 μM.

weaver–Burk type plot [$(\Delta A_{392-417})^{-1}$ versus $(\text{cholesterol}_{free})^{-1}$] showed that the spectral dissociation constant (K_s) for cholesterol binding was decreased slightly from 4.1 μM (native or $NaBH_4$ treated) to 2.8 μM (PLP modified) (data not shown). This may suggest that the PLP-modified enzyme has slightly higher binding affinity toward cholesterol than that of the native enzyme, provided that a difference extinction coefficient for cytochrome P-450$_{scc}$(SF) minus cytochrome P-450$_{scc}$ with saturating cholesterol concentration [i.e., $(\Delta A_{392-417})_{max}$] does not change upon this treatment. This observation indicates that the inhibition of the cholesterol side-chain cleavage activity of the cytochrome by the covalent PLP modification is not due to the lowered binding affinity toward substrate.

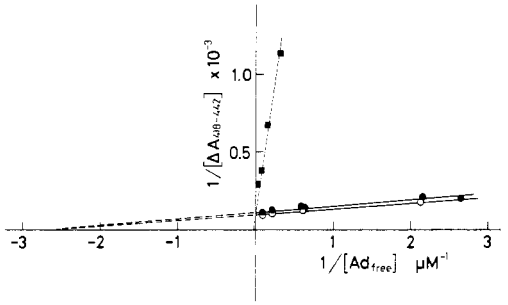

FIGURE 5: Effect of covalent PLP modification of cytochrome P-450_{scc}(SF) on its adrenodoxin binding affinity in substrate-free oxidized state. (●) Native cytochrome P-450_{scc}(SF); (○) $NaBH_4$-treated cytochrome P-450_{scc}(SF); (■) covalently PLP modified (2.0 mM PLP + $NaBH_4$) cytochrome P-450_{scc}(SF). Final cytochrome P-450_{scc}(SF) concentration was 2.83 μM.

Protection of Enzymatic Activity with Adrenodoxin against Inactivation by Covalent PLP Modification. To prove our hypothesis that an essential Lys residue(s) for the interaction with adrenodoxin is (are) selectively modified by the covalent PLP modification, we performed protection experiments using adrenodoxin as a protectant against the covalent PLP modification.

For the cholesterol side-chain cleavage assay, nearly 60% protection of original activity against the inactivation by the covalent PLP modification was observed when adrenodoxin (20% mole excess) was included during the incubation with PLP [Figure 4 (top)]. Addition of 0.35 M NaCl to the incubation mixture decreased the protecting effect of adrenodoxin to about half the original. For the electron-accepting activity assay, a similar protecting effect of adrenodoxin was observed [Figure 4 (bottom)]. Inclusion of 20% mole excess of adrenodoxin in the incubation mixture protected about 50% of the initial activity, and the addition of 0.35 M NaCl caused of the protecting effect of adrenodoxin to decrease significantly. This protecting effect is specific for adrenodoxin; inclusion of the same amount of bovine serum albumin (or spinach ferredoxin) with adrenodoxin (mole basis) during the covalent PLP modification of cytochrome P-450_{scc}(SF) did not show any protection against the inactivation (data not shown).

Effect of Covalent PLP Modification on Interaction of Cytochrome P-450_{scc} with Adrenodoxin in the Absence of Cholesterol. To see the direct effect of PLP treatment followed by $NaBH_4$ reduction upon the interaction of cytochrome P-450_{scc}(SF) with adrenodoxin, we carried out titration experiments with adrenodoxin in the absence of cholesterol. Optical perturbation of native (or PLP-modified) cytochrome P-450_{scc}(SF) heme upon adrenodoxin binding was analyzed by difference absorption spectroscopy with a four-cuvette assembly as described under Experimental Procedures. Upon addition of adrenodoxin to cytochrome P-450_{scc}(SF), difference spectra very similar to those of Lambeth et al. (1980) were produced. An apparent dissociation constant (K_s) for adrenodoxin binding was found to increase from 0.38 μM (native) to 33 μM (covalently PLP modified) (Figure 5). This observation proved that PLP treatment followed by $NaBH_4$ reduction of cytochrome P-450_{scc}(SF) resulted in the loss of ability to interact with adrenodoxin due to the selective modification of the essential Lys residue(s).

Effects of Covalent PLP Modification of Cytochrome P-450_{scc}(Chol) on Absorption Spectra and Enzymatic Activities. Inclusion of substrate (cholesterol) during the covalent PLP modification was carried out to test its possible protective effect against the inactivation of the enzyme. When cytochrome P-450_{scc}(Chol) was treated with PLP (2.0 mM) followed by

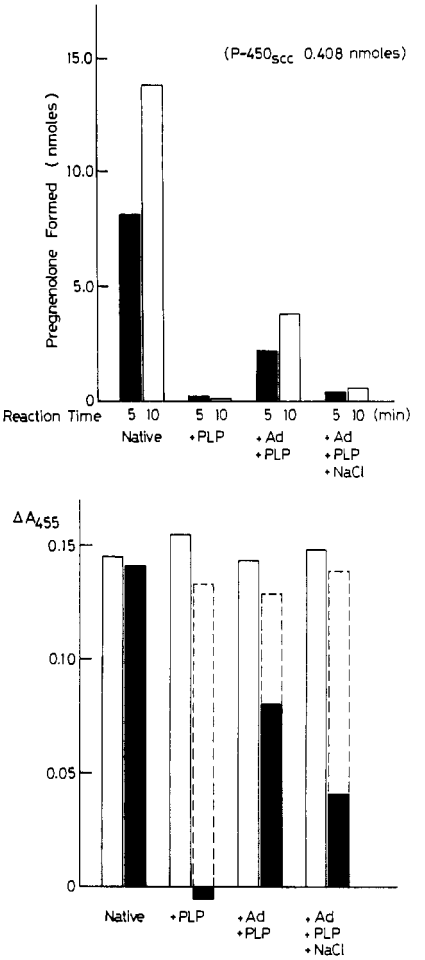

FIGURE 6: (Top) Inhibition of cholesterol side-chain cleavage activity by covalent PLP modification of cytochrome P-450_{scc}(Chol) (+PLP), protection of the activity by adrenodoxin (20% mole excess) against inactivation (+Ad + PLP), and inhibition of protection of activity by adrenodoxin in the presence of 0.35 M NaCl during the PLP modification. Side-chain cleavage reaction time: (solid bars) 5 min; (open bars) 10 min. (Bottom) Inhibition of electron-accepting activity by covalent PLP modification of cytochrome P-450_{scc}(Chol) (+PLP), protection of activity by adrenodoxin against inactivation (+Ad + PLP), and inhibition of protection of activity by adrenodoxin in the presence of 0.35 M NaCl during the PLP modification (+Ad + PLP + NaCl). Experimental conditions are the same as in Figure 4.

$NaBH_4$ reduction, its absorption spectrum showed a new band at 325 nm, characteristic of substituted pyridoxamine 5′-phosphate, just as for cytochrome P-450_{scc}(SF), and in addition an increase of the 392-nm-band intensity. The increase of the 392-nm-band intensity must be due to the slightly increased binding affinity of the PLP-modified enzyme toward cholesterol, as described before for cytochrome P-450_{scc}(SF). In the CO-reduced state, there was no indication of the formation of the P-420 form upon this treatment. The number of Lys residues covalently modified with PLP was estimated in the presence of detergents (Emulgen 913 and sodium cholate) and was found to be 3–4 residues/cytochrome P-450_{scc} molecule (spectra not shown).

The presence of cholesterol did not show any protective effect of the enzymatic activities (cholesterol side-chain cleavage and electron-accepting activities) of the cytochrome against the covalent PLP (2.0 mM) modification (Figure 6). Partial protection of original enzymatic activities against the inactivation by the covalent PLP modification was found when adrenodoxin (20% mole excess) was included during the incubation with PLP (Figure 6). Addition of 0.35 M NaCl to the incubation mixture decreased the protecting effect of

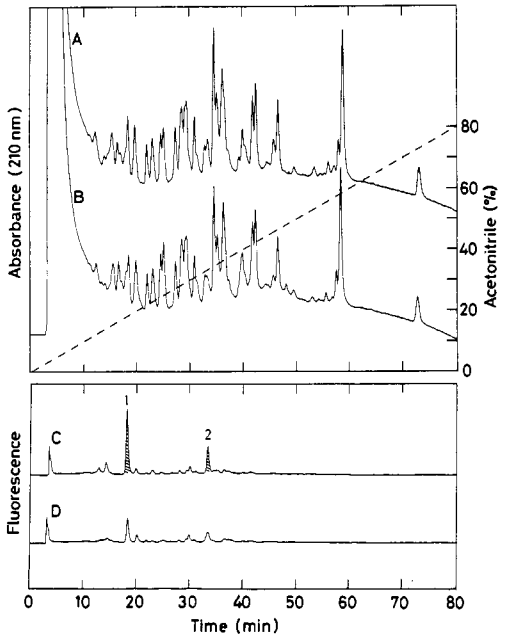

FIGURE 7: Comparison of HPLC tryptic peptide maps of cytochrome P-450$_{scc}$(SF) covalently modified with PLP (0.5 mM) in the absence (A and C) or presence (B and D) of adrenodoxin, monitored by absorbance at 210 nm (upper panel) and by fluorescence at 398 nm (with excitation at 298 nm) (lower panel).

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                 378                              385
Peptide 1:  Ala-Ser-Ile-Lys*-Glu-Thr-Leu-Arg

                 369                                        381
Peptide 2:  Met-Leu-Gln-Met-Val-Pro-Leu-Leu-Lys*-Ala-Ser-Ile-Lys
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FIGURE 8: Amino acid sequences of adrenodoxin-binding-site peptides of cytochrome P-450$_{scc}$. Lys 381 in peptide 1 and Lys 377 in peptide 2 (both marked with asterisks) were completely derivatized with pyridoxal 5′-phosphate (PLP).

adrenodoxin. All these observations are essentially the same as in the case of cytochrome P-450$_{scc}$(SF).

Identification of Adrenodoxin-Binding-Site Peptides. Figure 7 shows the HPLC elution profiles of the tryptic digests of cytochrome P-450$_{scc}$(SF) covalently modified with PLP (0.5 mM) in the presence and absence of adrenodoxin, respectively. Only two peptides were specifically labeled with PLP as indicated by fluorescence data at 398 nm (peptides 1 and 2, indicated by shading in the figure) when the covalent modification was carried out in the absence of adrenodoxin. These two peptides showed a dramatic decrease of fluorescence intensity upon the covalent PLP modification in the presence of 20% excess adrenodoxin. These fluorescent peptides were collected, lyophilized, rechromatographed, and characterized by automated Edman degradation. The amino acid sequences of these peptides are shown in Figure 8. Lys 381 in peptide 1 and Lys 377 in peptide 2 were found to be completely derivatized with pyridoxal 5′-phosphate (PLP).

We also tried a similar analysis for the tryptic digests of cytochrome P-450$_{scc}$(SF) covalently modified with PLP (2.0 mM) in the presence and absence of adrenodoxin, respectively. Although a selective fluorescent labeling of these two peptides was not so obvious under this condition, the presence of adrenodoxin during the covalent PLP modification caused a significant decrease of fluorescent intensity of these peptides.

The selective labeling of the unique Lys residues with PLP at 0.5 mM, the selective protection of the Lys residues by adrenodoxin against the covalent PLP modification, and the extent of the inactivation of electron-accepting activity under the same condition suggest that the region containing these unique Lys residues is responsible for the interaction with adrenodoxin.

Discussion

Inhibition of Electron Transfer by Covalent PLP Modification. On the basis of the model of cytochrome P-450$_{scc}$ catalytic cycle (Jefcoate, 1986), the inactivation of the cholesterol side-chain cleavage activity of cytochrome P-450$_{scc}$ might be caused by inhibition of (1) access of the substrate to the proper substrate-binding site, (2) introduction of the first electron from adrenodoxin, (3) binding of molecular oxygen to the heme, (4) introduction of the second electron from adrenodoxin; and/or (5) release of product from the enzyme.

Since the electron-accepting ability from adrenodoxin was mostly restored upon dilution or dialysis of the PLP-incubated enzyme and the treatment with $NaBH_4$ alone did not cause any effect on the enzymatic activity, the inactivation of the enzymatic activities by the covalent PLP modification is not due to an irreversible conformational change of the whole molecule. Considering the reaction condition (cytochrome P-450$_{scc}$ concentration, 200 μM; PLP concentration, 0–2.0 mM), the number of modified Lys residues after $NaBH_4$ reduction, and the extent of inactivation of the enzymatic activities at corresponding PLP concentration during the treatment (Figure 2), only a few Lys residues located at the adrenodoxin-binding site are selectively modified with PLP. This selectivity of the modification was manifested clearly in the protection experiments. Partial protection of the enzymatic activities (the electron-accepting activity and the cholesterol side-chain cleavage activity) were afforded by only 20% mole excess of adrenodoxin, but there was no protection of the activities when 20% mole excess bovine serum albumin (or spinach ferredoxin) was included instead during the PLP treatment. Bovine serum albumin (M_r = 67000; pI = 4.7–4.9) contains 59 Lys residues per molecule, while bovine adrenodoxin (M_r = 14000; pI = 4.0) has only 5 residues per molecule. On the other hand, spinach ferredoxin has the same pI value (4.0) as adrenodoxin. Thus, the protecting effect of adrenodoxin is not due to a dilution of PLP by its Lys residues nor to the nonspecific electrostatic binding of adrenodoxin to cytochrome P-450$_{scc}$.

One may suggest that a part of the inactivation of the cholesterol side-chain cleavage activity is caused by inhibition of release of product (pregnenolone) from the enzyme due to the slightly enhanced steroid-binding affinity or by disruption of productive substrate binding by the covalent PLP modification. However, the close parallelism of the electron-accepting ability and the cholesterol side-chain cleavage activity in the protection experiments and the absence of protecting ability of substrate (cholesterol) against the inactivation of the enzymatic activities by the covalent PLP modification seem to suggest that these possibilities are unlikely. But we could not rule out completely these possibilities, since we have not succeeded in isolating the enzyme singly modified at the adrenodoxin-binding site.

Adrenodoxin-Binding Site. Two identified adrenodoxin-binding-site peptides were found to correspond to peptides from residue 378 to residue 385 and from residue 369 to residue 381, respectively, of bovine cytochrome P-450$_{scc}$ (numbering system includes presequence to be cleaved during the translocation to mitochondria) (Morohashi et al., 1984) and showed an overlapping from residue 378 to residue 381. It is very interesting to note that only one of adjacent Lys residues either at position 377 or at position 381 was specifically modified

with PLP, and there was no peptide recovered in which both positions were modified with PLP. The region from Pro 374 to Arg 385 is located in one of the four highly conserved regions (i.e., C3 region) revealed by the comparison of the primary structures of bovine cytochromes P-450$_{scc}$ and P-450$_{11\beta}$ (Morohashi et al., 1987a). The primary structure of human cytochrome P-450$_{scc}$ shows sequence around this region identical with that of the bovine enzyme (Morohashi et al., 1987b). Both cytochromes P-450$_{scc}$ and P-450$_{11\beta}$ are mitochondrial inner membrane proteins and receive electrons from a common electron donor, adrenodoxin. Therefore, it is very likely that this highly conserved region is the common adrenodoxin-binding site of the mitochondrial cytochrome P-450 enzymes.

The precise mechanism of the inhibition of the electron-transfer activity by the covalent PLP modification remains to be elucidated. Probably, the conversion of the positively charged Lys residue(s) to bulky, negatively charged PLP–Lys residue(s) could account for the significant inhibition of the interaction with adrenodoxin, since the interaction between adrenodoxin and cytochrome P-450$_{scc}$ is thought to be dependent on multiple complementary charge pairs between these two proteins. It is very attractive to consider that Lys 377, Lys 381, and Arg 385 made charge pairs with Glu 74, Asp 79, and Asp 86 of adrenodoxin. There is a possibility that the selective reactivity of the formyl moiety of PLP toward the ϵ-amino group of Lys 381 (or Lys 377) to form a Schiff base arises from the electrostatic interaction between the negatively charged phosphate moiety of PLP and a cluster of positively charged amino acid residues such as Lys 377, Lys 381, and Arg 385.

Recently, Nelson and Strobel (1988) suggested that the interaction between two types of electron-donor proteins (i.e., iron–sulfur protein in mitochondrial system and flavoprotein in microsomal system) and their accompanying cytochromes P-450 is not very different on the basis of the comparison of 34 aligned cytochrome P-450 sequences. In other words, all cytochromes P-450 (bacterial, mitochondrial, and microsomal types) may interact with their electron-donor proteins via similar charged residues on their surfaces. Indeed, the identified adrenodoxin-binding site is located in the midst of the conserved regions suggested by Nelson and Strobel (1988) (alignment position 400–466), which may be involved in charge pairing between the reductase and the microsomal cytochrome P-450. Nelson and Strobel (1988) showed that Glu 382 and Arg 385 are fully conserved in all cytochromes P-450 examined by them. In addition, Ala 378, Ile 380, and Leu 376 are also well conserved in this region among various cytochromes P-450 examined. More interestingly, the sequence from Leu 375 to His 387 of cytochrome P-450$_{scc}$ corresponds to the sequence from Ile 281 to Phe 292 of cytochrome P-450$_{cam}$, which forms a short α-helix (K helix) in the proximal side of the heme according to X-ray crystallographic studies (Poulos et al., 1986, 1987). Probably, residues Lys 381 and Lys 377 (Arg in P-450$_{11\beta}$) in K helix may be important for the specificity toward adrenodoxin, since these residues are conserved only in mitochondrial enzymes (Morohashi et al., 1987a). Thus, the present results strongly support the suggestions made by Nelson and Strobel (1988).

Since all spectral indications in the present study suggested that the covalent PLP modification of the essential Lys residues (Lys 381 or Lys 377) for the specific binding with adrenodoxin did not cause any significant effect on the structure around the heme moiety (such as formation of the P-420 form), the adrenodoxin-binding site of the enzyme may not be located close to the heme. This view is in accordance with our recent EPR study on the interaction between the ferrous–NO complex of cytochrome P-450$_{scc}$ and reduced adrenodoxin (Tsubaki et al., 1988). We estimated in that study that the distance between the Fe_2S_2 core of reduced adrenodoxin and the ferrous–NO heme of the cytochrome in complexed form is more than 10 Å. Indeed, Tuls et al. (1987) reported recently that the heme group in cytochrome P-450$_{scc}$ is deeply buried at least 26 Å from the binding domain for adrenodoxin from their fluorescence energy transfer studies.

The binding affinity of adrenodoxin to cytochrome P-450$_{scc}$(SF) (K_d = 0.4 μM) is much weaker than that to cytochrome P-450$_{scc}$ in the presence of cholesterol (K_d = 0.07 μM) (Lambeth et al., 1980; Hanukoglu et al., 1981a). However, the binding strength to cytochrome P-450$_{scc}$(SF) is still strong enough to achieve the purification of cytochrome P-450$_{scc}$(SF) on an affinity column chromatography using adrenodoxin–Sepharose 4B (Tsubaki et al., 1986a). It has been thought that adrenodoxin binding to cytochrome P-450$_{scc}$(SF) causes a small spin-state change (increase from 0 to 15% high-spin component) on the basis of spectral perturbation in the Soret region (Lambeth et al., 1980). However, close examinations of our present data for native cytochrome P-450$_{scc}$(SF) and that of Lambeth et al. (1980) show that the spectral change in the Soret region upon adrenodoxin binding is a broadening of the Soret band of the low-spin state rather than a typical spin-state change (type II, from low to high spin). We conclude that this spectral change in the Soret region is caused by a structural change of the ferric heme moiety upon adrenodoxin binding in the absence of substrate. In the absence of substrate in the substrate-binding site of the enzyme, the tertiary structure around the heme moiety is not so rigid and cannot resist the trend of structural change upon adrenodoxin binding, leading to the broadening of the Soret band.

For an understanding of the molecular mechanism of the electron transfer from adrenodoxin to the heme iron of cytochrome P-450$_{scc}$, more detailed studies including the isolation of the enzyme singly modified with PLP at the adrenodoxin-binding site (i.e., Lys 381 or Lys 377) are necessary.

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