radical in liposomes (Scarpa et al., 1984; Doba et al., 1985; Niki et al., 1985; Liebler et al., 1986). Recycling of the tocopheroxyl radical may proceed more efficiently because there is no competing hydrolysis reaction as there is for T^+. Some form of biochemical catalysis would almost certainly be required for a two-electron redox cycle to efficiently regenerate TH from 8a-(alkyldioxy)tocopherones in living tissues. Since 8a-(alkyldioxy)tocopherones account for about half of the TH consumed by peroxyl radicals in lipid bilayers,[3] it seems plausible that their enzyme-catalyzed reduction to TH may occur.

ACKNOWLEDGMENTS

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Registry No. 1, 123438-35-7; TH, 59-02-9; TQ, 62726-91-4; AMVN, 4419-11-8; ascorbic acid, 50-81-7; 8a-hydroxy-α-tocopherol, 3626-81-1.

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Conformational Change of the Heme Moiety of the Ferrous Cytochrome P-450$_{scc}$–Phenyl Isocyanide Complex upon Binding of Reduced Adrenodoxin†

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ABSTRACT: Reduction of cytochrome P-450$_{scc}$(SF) (SF, substrate free) purified from bovine adrenocortical mitochondria with sodium dithionite ($Na_2S_2O_4$) or with β-NADPH mediated by catalytic amounts of adrenodoxin and adrenodoxin reductase in the presence of phenyl isocyanide produced a ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex with Soret absorbance maximum at 455 nm having a shoulder at 425 nm. On the other hand, when a preformed cytochrome P-450$_{scc}$(SF)–adrenodoxin complex was reduced chemically or enzymatically under the same conditions, the absorbance spectrum showed drastic changes, i.e., an increase in intensity at 425 nm and a concomitant decrease in intensity at 455 nm. Similar spectral changes could be produced by addition of the same amount of reduced adrenodoxin afterward to the ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex. Titration experiments with adrenodoxin showed that (1) a 1:1 stoichiometric saturation of the spectral change was obtained for both the absorbance increase at 425 nm and the absorbance decrease at 455 nm, (2) there was no spectral change in the presence of 0.35 M NaCl, and (3) there was no spectral change for cytochrome P-450$_{scc}$(SF) whose Lys residue(s) essential to the interaction with adrenodoxin had been covalently modified with PLP. These results suggest that ternary complex formation of ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide with reduced adrenodoxin caused a conformational change around the ferrous heme moiety. By analysis of temperature and pH dependencies of the spectral change of the ternary complex, it was suggested that this conformational change may reflect the essential step for electron transfer from reduced adrenodoxin to the ferrous–dioxygen complex of cytochrome P-450$_{scc}$.

Cytochrome P-450$_{scc}$ is an iron protoporphyrin IX containing monooxygenase, which is responsible for the side-chain cleavage reaction of cholesterol to produce pregnenolone, the first and rate-limiting step of steroid hormone biosynthesis (Burstein & Gut, 1971). The side-chain cleavage reaction consists of three consecutive monooxygenase reactions; each reaction can be envisioned as a cyclic process composed of five major steps and requiring two electrons and a dioxygen molecule. The steps comprising the first of this series of

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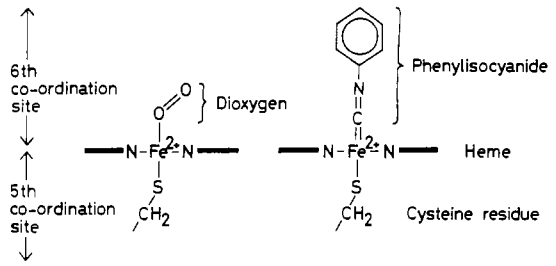


FIGURE 1: Schematic representation of the active site of ferrous cytochrome P-450 complexed with dioxygen (left) and with phenyl isocyanide (right).

reactions are, briefly, (1) binding of cholesterol to the ferric, low-spin, substrate-free cytochrome, resulting in the conversion of the heme iron from low to high spin; (2) introduction of the first electron to ferric cholesterol-bound cytochrome P-450_{scc}, yielding ferrous cholesterol-bound cytochrome; (3) dioxygen binding to the ferrous heme; (4) introduction of the second electron to oxyferrous cytochrome P-450_{scc}; and (5) a hydroxylation reaction proceeding with the insertion of one oxygen atom from the dioxygen into the substrate and reduction of the second oxygen atom to water. At this stage, the enzyme is ready to begin the second cycle of the reaction with the introduction of the second oxygen atom [see reviews such as Lambeth et al. (1982) and Jefcoate (1986)].

The two electrons required for the activation of dioxygen and the completion of one catalytic cycle are supplied by reduced adrenodoxin, a 2Fe-2S iron–sulfur protein that mediates the transfer of these electrons from β-NADPH and adrenodoxin reductase to the cytochrome (Orme-Johnson & Beinert, 1969; Estabrook et al., 1973). With respect to catalytic turnover, control of the cytochrome P-450_{scc} reaction cycle seems to occur at one of the electron-donation steps. In this context, the analysis of the interaction between reduced adrenodoxin and the ferrous cytochrome P-450_{scc}–dioxygen complex is one of the most important points for the understanding of the molecular mechanism of the cytochrome P-450_{scc} reaction.

Phenyl isocyanide is known as one of the specific inhibitors for various cytochromes P-450 (Ichikawa & Yamano, 1968). Its inhibition mechanism depends on its high affinity for the sixth ligand (dioxygen) binding site of the ferrous heme iron of cytochrome P-450 (Figure 1). This is a property which is common to many alkyl isocyanides and aromatic isocyanides (Mims et al., 1983; Wood et al., 1987). The reduction of cytochrome P-450 is followed by the binding of phenyl isocyanide (or alkyl isocyanide) to afford a ferrous cytochrome P-450–phenyl isocyanide (or alkyl isocyanide) complex, and this complex is stable even in aerobic conditions (Imai & Sato, 1968b; Tsubaki et al., 1986b). This ferrous heme–phenyl isocyanide (or alkyl isocyanide) complex cannot accept the second electron from the electron donor (ferredoxin for the mitochondrial system and cytochrome P-450 reductase for the microsomal system). This complex is suitable for analyzing the interaction between the electron donor in the reduced state and ferrous cytochrome P-450 in the ligated state. In addition, the absorbance spectra of the ferrous heme–phenyl isocyanide (or alkyl isocyanide) complexes exhibit two Soret peaks, and the relative intensities of these absorbance bands are sensitive to conformational changes around the heme moiety of the cytochrome P-450 induced by pH change (Imai & Sato, 1966, 1967) and by an ionic strength change (Imai & Sato, 1968a). The relative intensities are also dependent on the length of the hydrocarbon chain of alkyl isocyanides (Ichikawa & Yamano, 1968).

The present study was conducted to examine the possible role of the conformational change of the tertiary structure of the heme moiety of ferrous cytochrome P-450_{scc} in the ligated state upon an electron-transfer reaction from its physiological electron donor, reduced adrenodoxin.

Experimental Procedures

Materials. Cytochrome P-450_{scc}(SF), adrenodoxin reductase, and adrenodoxin were purified to homogeneity as previously described (Tsubaki et al., 1986a,b; Hiwatashi et al., 1976, 1986). Phenyl isocyanide was synthesized as described previously (Schmidt & Stern, 1929). Emulgen 913 was obtained from Kao-Atlas. Other chemicals were obtained from Sigma or Wako Pure Chemicals and used without further purification.

Cytochrome P-450_{scc}(SF) in 10 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.1 mM EDTA was stored as a stock solution (400–500 μM) and was diluted with 10 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1% (v/v) Emulgen 913, and 0.25% (w/v) sodium cholate (buffer A) upon use. Adrenodoxin in 10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA was stored as a stock solution (150–1000 μM) and was diluted with buffer A upon use.

Reduction of Cytochrome P-450_{scc}(SF) and Spectroscopic Measurements. Three milliliters of cytochrome P-450_{scc}(SF) (typically 3 μM final concentration) in a quartz cuvette was reduced in the presence of phenyl isocyanide (480 μM) by addition of a slight excess of sodium dithionite ($Na_2S_2O_4$) or by addition of β-NADPH (10 μL, 20 mM); in the latter case, catalytic amounts of adrenodoxin reductase (20 nM final concentration) and adrenodoxin (50 nM final concentration) were added to mediate the electron transfer. In some experiments, a 20% mole excess of adrenodoxin relative to cytochrome P-450_{scc} was added instead, and the concentration of adrenodoxin was also increased (70 nM final concentration). The cuvette containing the sample was placed in a temperature-controlled cell holder, and the time-dependent absorbance change and the absorbance spectra after completion of the reaction were recorded by a Shimadzu UV-240 spectrophotometer.

Titration with reduced adrenodoxin was performed at 25 °C by addition of concentrated adrenodoxin in the oxidized state to the sodium dithionite reduced ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex in a cuvette. The absorbance change at 455 or 425 nm was monitored with the spectrophotometer in the time-scanning mode; the oxidized adrenodoxin added was eventually reduced with excess sodium dithionite in the cuvette, and the resulting reduced adrenodoxin formed a ternary complex with the ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex.

The pH dependency of the spectral change of the ternary complex [ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complexes with reduced adrenodoxin] was analyzed in the range between pH 5.9 and pH 8.9 in 10 mM potassium phosphate buffer containing the same ingredients as buffer A at 19.7 °C. After each spectroscopic measurement, the pH value was checked.

Results

Addition of sodium dithionite ($Na_2S_2O_4$) to cytochrome P-450_{scc}(SF) in buffer A in the presence of phenyl isocyanide produced a ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex with a characteristic absorbance spectrum having a Soret maximum at 455 nm and a shoulder at 425 nm (Figure

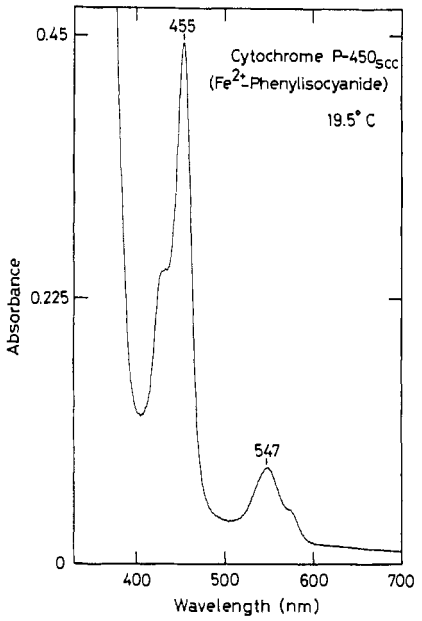
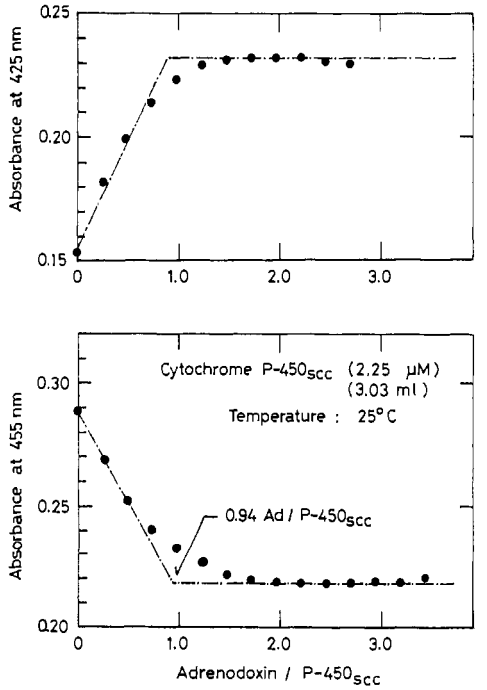


FIGURE 2: Visible absorbance spectrum of ferrous cytochrome P-450$_{scc}$(SF) complexed with phenyl isocyanide. Ferric cytochrome P-450$_{scc}$(SF) (3.18 μM) in buffer A was reduced with a slight excess of sodium dithionite in the presence of phenyl isocyanide at 19.5 °C.

2). The appearance of this new absorbance occurred with pseudo-first-order kinetics (data not shown).

If cytochrome P-450$_{scc}$(SF) was reduced with β-NADPH in the presence of catalytic amounts of adrenodoxin reductase and adrenodoxin to mediate electron transfer from the pyrimidine nucleotide to the hemoprotein, a similar ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex was formed. Further addition of sodium dithionite to the enzymatically reduced ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex showed a slight increase of 455-nm absorbance intensity, indicating that the heme of the cytochrome was almost (but not completely) reduced enzymatically with β-NADPH at pH 7.4. Since the midpoint oxidation–reduction potential of cytochrome P-450$_{scc}$(SF) is known to be around −412 mV, too negative to permit significant electron transfer from the physiological electron donor, adrenodoxin (Light & Orme-Johnson, 1981), the binding of phenyl isocyanide to ferric cytochrome P-450$_{scc}$(SF) must be accompanied by an increase in the oxidation–reduction potential, allowing for the electron transfer from adrenodoxin. The increase of the oxidation–reduction potential in the presence of phenyl isocyanide is not unusual, since the addition of a physiological substrate [such as cholesterol, 22(R)-hydroxycholesterol, etc.] to cytochrome P-450$_{scc}$(SF) causes an increase of the oxidation–reduction potential up to −265 to −285 mV (Lambeth & Kriengsiri, 1985), which is comparable to the midpoint potential of adrenodoxin (−273 to −291 mV) (Lambeth & Pember, 1983). Probably, phenyl isocyanide occupies the substrate binding site of the oxidized enzyme and expels a water molecule(s) at the active site, leading to the increase of the oxidation–reduction potential (Poulos et al., 1985, 1986, 1987; Raag & Poulos, 1989). (Indeed, phenyl isocyanide binding to the ferrous heme iron was inhibited considerably in the presence of cholesterol, and therefore, the present experiments were all performed in the absence of substrate for simplicity of the analysis.)

Addition of adrenodoxin to the sodium dithionite reduced ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex caused a spectral change, i.e., an increase of absorbance at 425 nm and a concomitant decrease of absorbance at 455 nm, in a dose-dependent manner. This spectral change eventually reached a maximum at an adrenodoxin:P-450$_{scc}$ = 1:1 ratio when the change was analyzed at 455 and 425 nm (Figure 3). The absorbance spectrum at this stage is presented in Figure 3 (right) in comparison with that before the addition of adrenodoxin. When the titration experiment with adrenodoxin was performed similarly in the presence of 0.35 M NaCl, there was no spectral change (Figure 4). This observation is reasonable, since the interaction between adrenodoxin and cytochrome P-450$_{scc}$ is predominantly electrostatic and is strongly inhibited by ions (Lambeth et al., 1979; Hanukoglu et al., 1981; Lambeth & Kriengsiri, 1985). There was no change



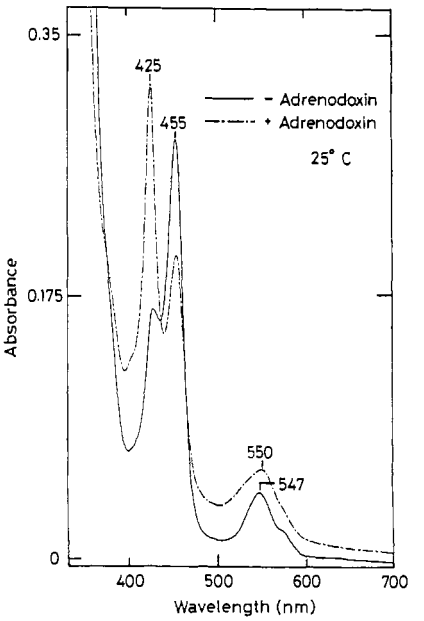


FIGURE 3: Titrations of the sodium dithionite reduced ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex (2.246 μM) with adrenodoxin (166.2 μM). (Left upper panel) Absorbance change at 425 nm; (left lower panel) absorbance change at 455 nm. (Right panel) Absorbance spectra of the ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex before (—) and after (-·-) the titration. The titrations were performed at 25 °C.

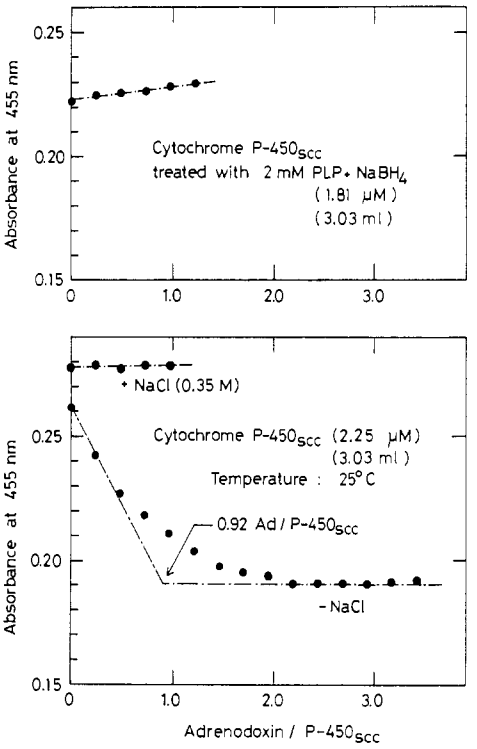


FIGURE 4: Inhibition of the absorbance spectral change of the ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex upon addition of reduced adrenodoxin by inclusion of 0.35 M NaCl (lower panel) and by pretreatment of cytochrome P-450_{scc} with pyridoxal 5′-phosphate (PLP)/$NaBH_4$ (upper panel). The titration was performed at 25 °C, and the spectral change was monitored at 455 nm.

in the absorbance spectrum of the ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex upon addition of 0.35 M NaCl alone. Likewise, there was no spectral change upon the addition of adrenodoxin to the sodium dithionite reduced ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex whose Lys residue(s) essential to the interaction with adrenodoxin had been covalently modified with pyridoxal 5′-phosphate (PLP) (Tsubaki et al., 1989) as shown in Figure 4. All these results suggest that the spectral change of the ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex upon addition of adrenodoxin is due to the conformational change of the ferrous heme–phenyl isocyanide moiety caused by the association of reduced adrenodoxin to the adrenodoxin binding site of the cytochrome. It is highly unlikely that the spectral change is due to the release of phenyl isocyanide from the ferrous heme on the basis of the spectral dissimilarity to the reduced form of the cytochrome. To simplify the discussion of these two spectral forms, we have called the one which has a Soret aborbance peak at 455 nm conformer I and the form with the peak at 425 nm conformer II.

When a cytochrome P-450_{scc}(SF)–adrenodoxin complex in the presence of phenyl isocyanide [this complex was formed in advance by mixing 20% mole excess of oxidized adrenodoxin with oxidized cytochrome P-450_{scc}(SF)] was enzymatically reduced with β-NADPH via a catalytic amount of adrenodoxin reductase, the resulting absorbance changes (monitored at 455 and 425 nm) were found to be composed of two phases as shown in Figure 5. There was a rapid phase which terminated within 30 s after addition of β-NADPH and a slow phase which continued for more than 5 min under these experimental conditions. The former phase seemed to correspond to the formation the ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex (conformer I), i.e., the heme reduction process. The hypothesis that the initial species formed on reduction was conformer I was tested by determining the time dependence of the spectral changes. This time-dependent change was analyzed by plotting the absorbance intensity at various wavelengths in the Soret region at 30 s after addition of β-NADPH (Figure 6), showing a clear resemblance. The latter phase may correspond, therefore, to the association process of reduced adrenodoxin to the ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex to form conformer II since the spectrum after the completion of the second phase is very similar to that of the ferrous cytochrome P-450_{scc}-(SF)–phenyl isocyanide complex after complexation with reduced adrenodoxin (Figure 3). After completion of the second phase, the sample in the cuvette was further treated by addition

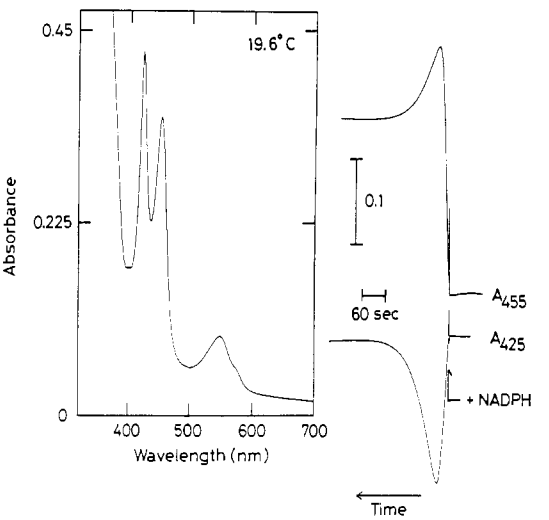


FIGURE 5: Enzymatic reduction of cytochrome P-450_{scc}(SF) with β-NADPH mediated by a catalytic amount of adrenodoxin reductase (70 nM final concentration) and an excess of adrenodoxin (20% mole excess) in the presence of phenyl isocyanide. Absorbance changes at 455 and 425 nm were monitored with the time-scan mode (right panel); the absorbance spectrum after completion of the reaction is shown (left panel). The reaction was performed at 19.6 °C.

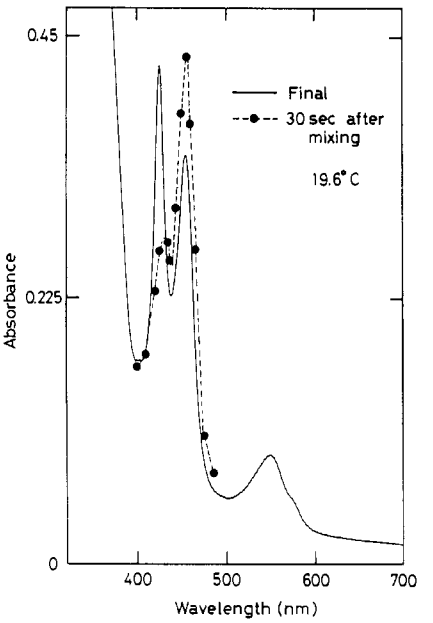


FIGURE 6: Analysis of an intermediate formed during the enzymatic reduction of cytochrome P-450_{scc}(SF) with β-NADPH mediated by a catalytic amount of adrenodoxin reductase (70 nM final concentration) and excess of adrenodoxin (20% mole excess) at 19.6 °C. Absorption intensities at various wavelengths 30 s after the mixing with β-NADPH were plotted in comparison to the spectrum after the completion of the reaction.

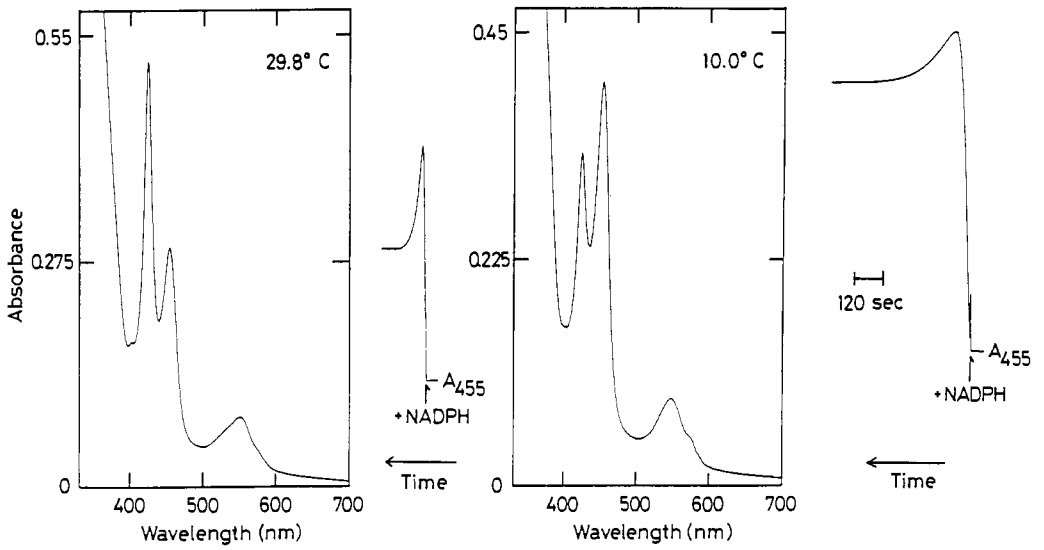


FIGURE 7: Temperature dependence for the formation of the ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide reduced adrenodoxin ternary complex. Cytochrome P-450$_{scc}$(SF) was enzymatically reduced with β-NADPH under the same conditions as in Figure 4, except for temperature. The right portion of the figure shows the reaction at 10.0 °C, whereas the left portion shows the reaction at 29.8 °C. Time-scanning (at 455 nm) speeds are identical for both reactions. The spectra after the completion of the reaction are also presented.

of sodium dithionite. But there was not additional spectral change, suggesting that cytochrome P-450$_{scc}$(SF) and adrenodoxin were fully reduced with adrenodoxin reductase and β-NADPH.

We then analyzed the temperature dependency of the spectral change of the ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex upon the association with reduced adrenodoxin. There was no temperature-dependent spectral change at all for the complex in the absence of adrenodoxin (below 30 °C, at pH 7.4). The heme reduction process of cytochrome P-450$_{scc}$(SF) (i.e., the first phase to form conformer I) was found to be temperature dependent, the higher the temperature, the faster the rate, as expected. More importantly, the rate and the extent of the spectral change in the second phase (the formation of conformer II upon reduced adrenodoxin binding) was suppressed at 10.0 °C considerably, compared with those at 19.8 °C (Figure 7). On the other hand, at 29.8 °C, the rate and the extent of absorption change in the second phase were much larger than those at 19.8 °C (Figure 7).

We then analyzed temperature-dependent absorbance spectra changes of the ternary complex [ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complexed with reduced adrenodoxin]. First, the ferrous ternary complex was formed at 29.8 °C in a cuvette as described under Experimental Procedures; then, the temperature of the sample was changed up and down, and the absorbance spectra were recorded at various temperatures as indicated in Figure 8. The facts that the spectra in Figure 8 showed a clear isosbestic point at 433 nm and that the temperature-dependent spectral change was fully reversible suggest the existence of a temperature-dependent conformational equilibrium between conformer I and conformer II in the ferrous heme within the ternary complex; i.e., at higher temperature the conformer II is favored whereas at lower temperature conformer I is stabilized.

Finally, we analyzed the pH-dependent spectral change of the ferrous ternary complex. It has been known that microsomal cytochrome P-450 shows a reversible pH-dependent spectral change when various alkyl isocyanides were bound to the ferrous heme (Imai & Sato, 1966, 1967; Ichikawa & Yamano, 1968). However, in the absence of adrenodoxin, the ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex did not show any spectral change at all between pH 7.0 and pH 9.0 and seemed to remain as conformer I in this region

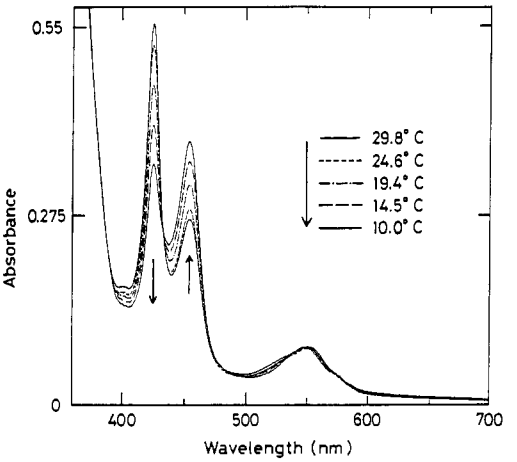


FIGURE 8: Temperature-dependent spectral change of the ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide reduced adrenodoxin complex. First, cytochrome P-450$_{scc}$(SF) was reduced with β-NADPH under the same conditions as in Figure 5, except for temperature, which was 29.8 °C, and its absorbance spectrum was recorded. Then the temperature of the sample was decreased very slowly so as to achieve equilibrium of the two conformers, and absorbance spectra were recorded. To exclude the reoxidation of the heme during the experiment, which continued for several hours, a slight amount of sodium dithionite was added, and the cuvette was sealed with Parafilm.

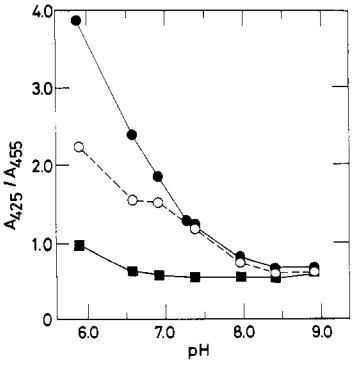


FIGURE 9: Influence of pH on the absorbance intensity ratio (A_{425}/A_{455}) for the ferrous cytochrome P-450$_{scc}$–phenyl isocyanide complex in the presence (●) and the absence (■) of reduced adrenodoxin. Plots for the enzymatically reduced (with β-NADPH) ferrous cytochrome P-450$_{scc}$–phenyl isocyanide reduced adrenodoxin ternary complex (○) are also presented for comparison.

(Figure 9). In the presence of reduced adrenodoxin, however, the resulting ferrous ternary complex exhibited a strong pH-dependent spectral change (Figure 9). This pH dependency is not due to weakening of the interaction between adrenodoxin and the cytochrome according to a report by Jefcoate et al. (1982) that a decrease in pH from 8.0 to 6.0 has no effect on adrenodoxin binding to cytochrome P-450_{scc} in the absence of substrate. At higher pH, above 8.0, the spectral change upon binding with reduced adrenodoxin was suppressed, and conformer I dominated; at lower pH, the spectral change was enhanced, and the equilibrium was shifted toward conformer II. This pH-dependent spectral change observed for the ferrous ternary complex was similar to many previous results obtained for various microsomal cytochromes P-450 (Imai & Sato, 1966, 1967; Ichikawa & Yamano, 1968). It must be noted that at lower pH, below 7.0, cytochrome P-450_{scc} could not be reduced completely with β-NADPH via adrenodoxin (20% mole excess) and adrenodoxin reductase as evidenced by the departure of the A_{425}/A_{455} ratios from those reduced with sodium dithionite (Figure 9).

Discussion

The tertiary structure of the active site of ferric cytochrome P-450_{scc} is known to be modified by the binding of oxidized adrenodoxin in the presence of cholesterol (Tsubaki et al., 1986a). Oxidized adrenodoxin not only increases the affinity of ferric cytochrome P-450_{scc} for cholesterol but induces a conformational change of the cytochrome even in the absence of substrate (Lambeth et al., 1980; Hanukoglu et al., 1981a; Tsubaki et al., 1989). Recently, an interaction between reduced adrenodoxin and a ferrous cytochrome P-450_{scc}–nitric oxide complex in the presence and the absence of substrate was investigated by EPR spectroscopy (Tsubaki et al., 1988). It was found in that study that binding of reduced adrenodoxin caused a structural change at the active site of the cytochrome both in the presence and in the absence of cholesterol.

Unusual spectra having two Soret peaks at 455 and 425 nm, upon combination with various alkyl isocyanides (or aromatic isocyanides, including phenyl isocyanide), have been reported for the reduced state of cytochromes P-450 in liver microsomes or purified cytochromes P-450 (Imai & Sato, 1966, 1967, 1968a; Ichikawa & Yamano, 1968; Griffin & Peterson, 1971). The relative intensities of these absorbance bands are reversibly affected by the nature of the buffer medium. Therefore, it has been suggested that the 455- and the 425-nm absorbance maxima represent two different isocyanide complexes of reduced cytochrome P-450, which exist in equilibrium (Imai & Sato, 1966). However, the structural basis of the difference between the two conformers, the 455-nm species and the 425-nm species, is unknown, although several hypotheses have been proposed (Imai & Sato, 1968c; Ichikawa & Yamano, 1968; Griffin & Peterson, 1971).

In the present study, first a 1:1 stoichiometric alteration of the absorbance spectrum of the ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex (reduced with sodium dithionite) upon addition of reduced adrenodoxin was found. The change in the absorbance spectrum must be a reflection of the conformational change of the ferrous heme moiety caused by the binding of reduced adrenodoxin to the adrenodoxin binding site of the cytochrome. This suggestion was confirmed by the absence of the spectral change in the titration experiments (1) when the titration was performed in the presence of 0.35 M NaCl, where the electrostatic interaction between adrenodoxin and the cytochrome was abolished (Lambeth et al., 1979; Hanukoglu et al., 1981b), and (2) when the PLP-modified cytochrome P-450_{scc} whose Lys residues are essential to the interaction with adrenodoxin were modified was used in the titration (Tsubaki et al., 1989). The existence of detergents (Emulgen 913 and sodium cholate) was found to be also essential to cause the alteration of the absorbance spectrum of the ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex upon addition of reduced adrenodoxin. In the absence of the detergents the spectral change was suppressed, and there was no clear 1:1 stoichiometry. This phenomenon may be explained as follows: in the absence of proper detergents cytochrome P-450_{scc} forms a large aggregate (Shikita & Hall, 1973a,b), and hydrophobic intermolecular forces may constrain each other and suppress the conformational change of the heme moiety upon binding of adrenodoxin or may inhibit the binding of adrenodoxin.

Second, we observed in the present study that there were two phases of absorbance change during the enzymatic reduction of the cytochrome P-450_{scc}(SF)–adrenodoxin complex in the presence of phenyl isocyanide (Figures 5 and 6). We have assigned the initial phase of absorbance changes to the formation of conformer I [ferrous cytochrome P-450_{scc}-(SF)–phenyl isocyanide complex] and the second phase to the formation of conformer II [ferrous cytochrome P-450_{scc}-(SF)–phenyl isocyanide complexed with reduced adrenodoxin] on the basis of spectral analysis. For our hypothesis to be correct, the following would have to occur:

$$Ad_r + \text{P-}450_{scc}{}^{3+} \xrightarrow{1} Ad_r\cdot\text{P-}450_{scc}{}^{3+} \xrightarrow{2} Ad_o\cdot\text{P-}450_{scc}{}^{2+} \xrightarrow{3} Ad_o + \text{P-}450_{scc}{}^{2+}$$

$$Ad_o + AdR_r \xrightarrow{4} Ad_r + AdR_o$$

$$Ad_r + \text{P-}450_{scc}{}^{2+} \xrightarrow{5} Ad_r\cdot\text{P-}450_{scc}{}^{2+}$$

where Ad_r is reduced adrenodoxin, Ad_o is oxidized adrenodoxin, AdR_r is reduced adrenodoxin reductase, and AdR_o is oxidized adrenodoxin reductase. In this scheme the binding of phenyl isocyanide to the ferrous heme at the stage of reaction two is neglected for simplicity. The rate-limiting step for the second phase would have to be either reaction 3, 4, or 5. To determine the rate-limiting step precisely in the apperance of the 425-nm form (conformer II), more systematic analyses would be necessary.

We feel that the conformational change of the ferrous heme moiety upon binding of reduced adrenodoxin as manifested in the phenyl isocyanide complex of cytochrome P-450_{scc} may be a common nature of mitochondrial cytochromes P-450 and, therefore, may play an important role in the physiological electron-transfer reaction (Tsubaki et al., 1988). A mechanism to explain the present observations is presented diagrammatically in Figure 10. This model is speculative, and there may be other possibilities to account for the present data; but this will serve as a working model for future investigations. In this model we assume that the overall tertiary structure of the ferrous cytochrome P-450_{scc}(SF)–phenyl isocyanide complex is not so much different from that of a ferrous cytochrome P-450_{scc}(SF)–dioxygen complex. We further assume that (1) there are two types of conformers (conformers I and II) in equilibrium for the structure of the ferrous heme–dioxygen moiety of cytochrome P-450_{scc} also when reduced adrenodoxin is bound to the cytochrome to transfer the second electron and (2) only one of the two conformers (i.e., conformer I) is favored in the absence of reduced adrenodoxin (Figure 10). It is very attractive to consider that one of the conformers (conformer II) is a suitable conformation for the second electron-transfer reaction from reduced adrenodoxin; the other (conformer I) is not. These two conformers are in temperature-dependent equilibrium; i.e., at higher temperature, conformer I is favored

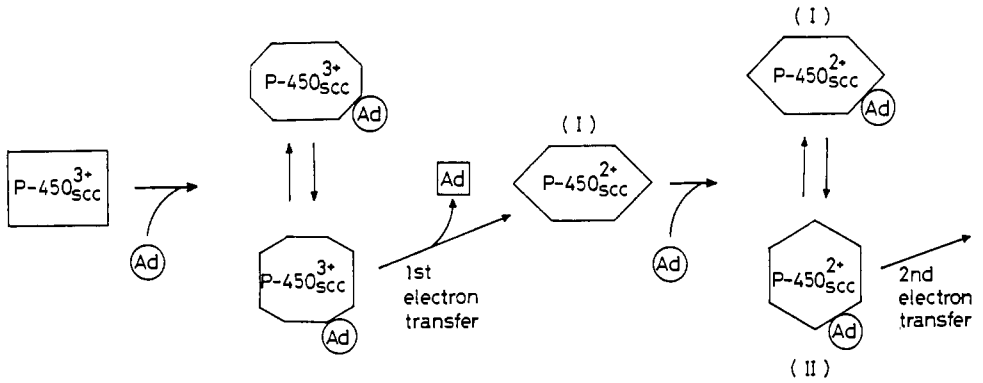


FIGURE 10: Schematic diagram of the electron-transfer reactions between cytochrome P-450$_{scc}$ and adrenodoxin. Ad in a circle indicates reduced adrenodoxin, whereas Ad in a square designates oxidized adrenodoxin. I and II refer to conformer I and conformer II, respectively.

while at low temperature conformer II is favored. These two conformers are also in pH-dependent equilibrium. It is reported that the rate-limiting step of the electron transfer from reduced putidaredoxin to cytochrome P-450$_{cam}$ is an intracomplex (cytochrome P-450$_{cam}$–putidaredoxin complex) conformational change rather than formation of a dienzyme complex (Hintz & Peterson, 1981; Brewer & Peterson, 1986, 1988). Therefore, the intracomplex conformational change from conformer I to conformer II may be an important step to transfer the electron from reduced adrenodoxin to the ferrous cytochrome P-450$_{scc}$(SF)–dioxygen complex. In the physiological reaction cycle there is no accumulation of conformer II, but in the presence of phenyl isocyanide and an excess of reduced adrenodoxin, conformer II could be accumulated and observed spectroscopically.

We have no definitive idea about the details of the structural difference between conformers I and II. But the intracomplex conformational change must be accompanied by the conformational change of reduced adrenodoxin itself in addition to that of the cytochrome itself, and such a intracomplex conformational change must be interdependent. A similar intracomplex mechanism may be also operative during the first electron transfer (Hinstz & Peterson, 1981; Brewer & Peterson, 1988) as depicted in Figure 10. Indeed, these structural changes are manifested in the shifts of the midpoint potentials of adrenodoxin and cytochrome P-450$_{scc}$ (from −273 to −291 mV for adrenodoxin and from −284 to −314 mV for cytochrome P-450$_{scc}$–substrate complex) upon intracomplex formation (Lambeth & Pembr, 1983).

It is noteworthy to report that when cytochrome P-450$_{11\beta}$ purified from bovine adrenocortical mitochondria was examined by the same way as for cytochrome P-450$_{scc}$ (i.e., reduced with β-NADPH via adrenodoxin reductase and excess adrenodoxin), a similar time-dependent spectral change composed of two phases, one being a rapid reduction of the heme and the other being a slow decrease of the 455-nm absorbance intensity, was observed. Therefore, the conformational change of the heme moiety of the ferrous ternary complex may be a common property for the mitochondrial cytochrome P-450 system.

One may raise a question: why no such spectral change occurred upon addition of reduced adrenodoxin to a ferrous cytochrome P-450$_{scc}$(SF)–carbon monoxide complex? The answer for this question is not clear at this stage; probably the bulkiness of the ligand is the most important factor. When carbon monoxide is bound to the ferrous heme iron in the absence of substrate, there may be no influence of the tertiary structure change of the cytochrome P-450$_{scc}$ caused by the binding of reduced adrenodoxin on the heme-bound carbon monoxide due to the relatively large heme pocket of the cytochrome (Poulos et al., 1985, 1986, 1987) and the smallness of the ligand. It is very interesting to examine whether such an intramolecular conformational change occurs in microsomal enzymes or not. Studies of this kind are underway in this laboratory.

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High-Resolution Differential Scanning Calorimetric Study of Myosin, Functional Domains, and Supramolecular Structures†

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ABSTRACT: High-resolution differential scanning calorimetry (DSC) has been employed to study the thermal stability of myosin, its major constitutive fragments (S-1, light chains, and rod), and also reconstituted thick filaments. The thermal denaturation of soluble myosin was complex and was characterized by a multistep endothermic process for the temperature range from 41 to 60 °C. The shape of the endotherm was highly dependent on the pH and the ionic strength of the solution, although the ΔH_{cal} (calorimetric enthalpy) of denaturation (1715 ± 75 kcal/mol) was insensitive to these changes for the solvent conditions used in this study. This value also agrees, within experimental error, with the sum of the denaturation enthalpies obtained for isolated fragments (1724 ± 79 kcal/mol). In identical conditions of ionic strength, pH, and heating rate, the computer-calculated differential endotherms of domains belonging to S-1 and light chains were superimposable with those of the isolated fragments. Their responses to changes in the solvent condition were also similar. We suggest that the observed functional independence of the major domains in myosin reflects also the independence of their structural stability. The thermal unfolding of the isolated rod was multiphasic and readily reversible (95%). It occurred between 41 and 60 °C, with an ΔH_{cal} of 1058 ± 59 kcal/mol. The melting of S-1 showed a single peak at 46.3 ± 0.1 °C with an ΔH_{cal} of 255 ± 12 kcal/mol. Light chains melted at 51.0 ± 0.2 °C with an ΔH_{cal} of 85 ± 15 kcal/mol. Despite the reversibility of the rod, the thermal denaturation of myosin was irreversible. When the ionic strength of the solution was gradually reduced to obtain synthetic filaments, the transition temperatures of domains converged, and the shape of the endotherm appeared sharp, with little effect on the overall enthalpy of denaturation (ΔH_{cal} of 1679 ± 98 kcal/mol). The peak of the overall transition of myosin filaments was 45 °C in 0.2 M KCl and increased to 49 °C in 0.1 M KCl. Electron micrographs showed different morphologies for filaments grown in an imidazole buffer and that grown in a phosphate buffer although the endotherms of these samples appeared similar. pH had a strong effect on the endotherms of these filaments. Lowering the pH from 7.0 to 6.7 in 0.1 M KCl increased the T_m of the main peak by 1.4 °C, resulting in the separation of the endotherm into two distinct transitions. These two transitions were broad, and their ΔH_{vH} values were much smaller than the ΔH_{cal}. Analysis suggests that the structural domains that constitute a myosin molecule also exist in these filaments and that the stability of these structural domains is equally sensitive to modulation by the ionic strength and the pH of the solution, consistent with the possibility of local structure events in the molecular mechanisms of muscle contraction.

In the attempt to elucidate the molecular mechanisms of muscle contraction, great emphasis has been given to the study of the thermal stability of myosin. Myosin is a chimeric protein. The globular heads (S-1) retain both its ATPase activity and the ability to move actin cable in vitro (Harada et al., 1987) even when they are separated from the fibrous tails in which the aggregation properties of myosin are located (Young et al., 1963). The tendency for myosin to coagulate during heat denaturation (Lowey & Holtzer, 1959) has limited the study with optical spectroscopies, such as absorption,

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