FORMATION OF A CYTOCHROME P-450$_{scc}$-ADRENODOXIN COMPLEX

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Summary: A cytochrome P-450 (P-450$_{scc}$), the substrate- and oxygen-binding component of bovine adrenal cortex mitochondrial cholesterol desmolase system forms a stable complex with adrenal ferredoxin (adrenodoxin). The complex formation is indicated either by a change of the absorption spectrum of P-450$_{scc}$ or, more directly, by gel filtration and density gradient centrifugation techiniques. The molar ratio of P-450$_{scc}$ and adrenodoxin is found to be 1:1 and the spectral dissociation constant for the binding, 160 nM. The binding can be induced only in the presence of bound cholesterol to P-450$_{scc}$. Thermal inactivation experiments provide additional evidence that indicates P-450$_{scc}$ combines with adrenodoxin to form a complex having a more rigid structure than P-450$_{scc}$.

The cholesterol desmolase activity of adrenal cortex mitochondria can be catalyzed by a reconstituted system composed of purified adrenodoxin (a ferredoxin), adrenodoxin reductase (an NADPH-dependent FAD-protein) and P-450$_{scc}$ (a P-450 cytochrome). All components are absolutely required for the conversion of cholesterol to pregnenolone and isocaproic aldehyde in the presence of NADPH and molecular oxygen, thereby suggesting formation of a steroid hydroxylase multienzyme complex among the three protein species.

A 1:1 complex of adrenodoxin with adrenodoxin reductase has been reported to be a catalytically active species forming electron transport chain from NADPH to the P-450, the substrate specific oxygen binding component (1-4). Efforts have also been made to determine whether or not P-450$_{scc}$ forms a complex either with adrenodoxin or adrenodoxin reductase alone, or with adrenodoxin and its reductase in the complexed form. However, the previous attempts have been uniformly unsuccessful (1). There is evidence that indicates a cytochrome P-450 (P-450$_{cam}$), isolated from Pseudomonas putida, forms a weakly bound 1:1 complex with putidaredoxin, the corresponding ferredoxin (5).

Recently we have separated and purified two different kinds of P-450 from bovine adrenal cortex mitochondria (6-8). One of these is P-450$_{scc}$

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which is specific in the conversion of cholesterol to pregnenolone. The P-450$_{scc}$ preparation is completely free from the other P-450 (P-450$_{11\beta}$) in respect of the enzyme activity, the spectral shift induced by substrate and immunological properties (9). With the P-450$_{scc}$ preparation obtained in the homogeneous state, binding of adrenodoxin to the P-450 was re-investigated. The present paper deals with the demonstration of the formation of tightly bound and stable adrenodoxin-P-450$_{scc}$ 1:1 complex in an effort to resolve the problem of the structural and functional organization of the desmolase system.

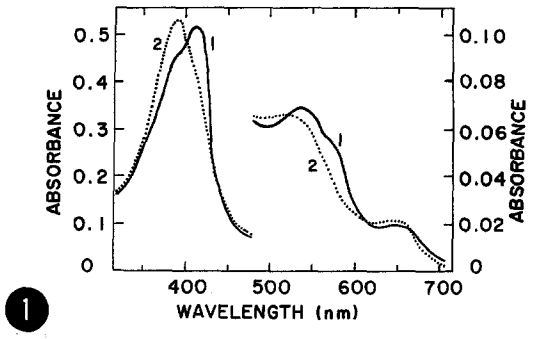
MATERIALS AND METHODS

Bio-gel P-300 (Bio·Rad), crystallized bovine serum albumin (Sigma) and NADPH (Boehringer) were purchased from the indicated sources. Other chemicals used were reagent grade. Crystalline bovine adrenodoxin (10), adrenodoxin reductase (11) and P-450$_{scc}$(6, 12) were prepared and assayed as previously described unless otherwise noted. The P-450$_{scc}$ preparation contained cholesterol (0.7-0.9 mole/mole of P-450 heme). Cholesterol-free P-450$_{scc}$ and the reconstituted cholesterol-bound P-450$_{scc}$ were prepared by modification of methods as described elswhere (6, 12). Spinach ferredoxin and putidaredoxin were prepared and estimated according to the methods of Buchanan and Arnon (13) and Tagawa and Arnon (14), and Cushman *et al.* (15), respectively. Adrenodoxin and spinach ferredoxin were also assayed by measuring their effects on the rate of increase of absorbances; adrenodoxin, at 550 nm of cytochrome *c* in a mixture containing NADPH, adrenodoxin reductase and cytochrome *c* (11), and spinach ferredoxin, at 448 nm of P-450$_{scc}$ in the presence of dithionite, carbon monoxide and P-450$_{scc}$ (16), respectively.

Absorbance measurements were made on a temperature controlled Union model SM-401 recording spectrophotometer with a cuvette of 1-cm pathlength. Density gradient centrifugation was carried out in 4.8 ml of 5-20 % w/v sucrose gradients containing 50 mM potassium phosphate buffer, pH 7.4. Samples (200 µl) were layered on the top of the gradients and centrifuged for 15 hrs at 45,000 rpm at 5° in a SW 50L rotor using a Beckman L5-75 ultracentrifuge.

RESULTS

The spectrum of the purified preparation of P-450$_{scc}$ exhibited splitting of the Soret peak (393 nm) in a certain range of temperature (17), pH (18, 19) and ionic strength (18), though the nature of the spectral changes is not yet fully understood. We found that the presence of adrenodoxin has remarkable effects on the spectrum (Fig. 1). An increase in temperature at a neutral pH caused a decrease in the absorbance of P-450$_{scc}$ at 393 nm, with concomitant increases around 420 nm, 530 nm and 570 nm. The absolute spectrum shown in Fig. 1, *curve 1* represents the spectrum of oxidized P-450$_{scc}$ in 10 mM potassium phosphate buffer, pH 7.3, at 37°. When adrenodoxin was added to the P-450$_{scc}$, the difference in the Soret region of the P-450 increased at 393 nm and decreased at 418 nm (Fig. 1, *curve 2*). Similar but



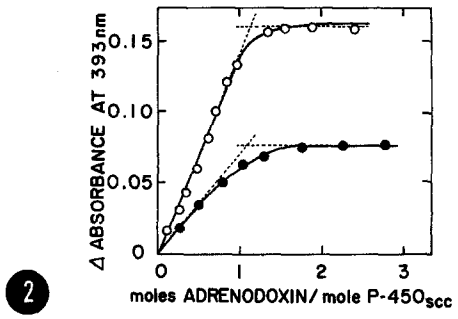


Fig. 1. The effect of adrenodoxin on the spectrum of P-450_{scc} dissolved in 10 mM potassium phosphate buffer, pH 7.3, at 37°. Curve 1 (———), 6 μM P-450_{scc}, and curve 2 (·····), 6 μM P-450_{scc} plus 15 μM adrenodoxin; the reference cuvette contained adrenodoxin in the same buffer.

Fig. 2. Spectrophotometric titration of P-450_{scc} by incremental additions of adrenodoxin. The reaction mixture contained 12 μM P-450_{scc} (-o-), or 5.8 μM P-450_{scc}(-●-), and indicated amounts of adrenodoxin in 10 mM potassium phosphate buffer, pH 7.4, at 37°. The reference cuvette contained adrenodoxin in the same buffer.

a less pronounced effect was induced when either spinach ferredoxin or putidaredoxin instead of adrenodoxin was used, while no appreciable change was observed by bovine serum albumin. These results suggest the formation of a new complex between P-450_{scc} and adrenodoxin.

Since the changes in absorption were indicated at 393 nm and thus the stoichiometry of the components of the complex could be determined spectophotometrically, titration of the P-450_{scc} with sequential addition of adrenodoxin was carried out (Fig. 2). The resultant stoichiometry was 1 mole adrenodoxin per 1 mole of P-450. Assuming that the increases in absorption at 393 nm are proportional to the amount of the complex, a mean value of the spectral dissociation constant of the complex was calculated to be 160 nM. These results indicate that the two proteins make a tightly bound 1:1 complex.

The interaction of P-450_{scc} with adrenodoxin, indicated above by the absorbance change, was further confirmed from different lines of evidence, including direct separation and characterization of the complex. Fig. 3, a-e illustrates the binding verified by sucrose density gradient ultracentrifugation at 5° in 50 mM potassium phosphate buffer, pH 7.4. The results reveal that a nearly equivalent amount of adrenodoxin is bound with P-450_{scc}. Spinach ferredoxin could incompletely replace adrenodoxin (Fig. 3, f). When a similar experiment was carried out with an equimolar mixture of P-450_{scc} and adreno-

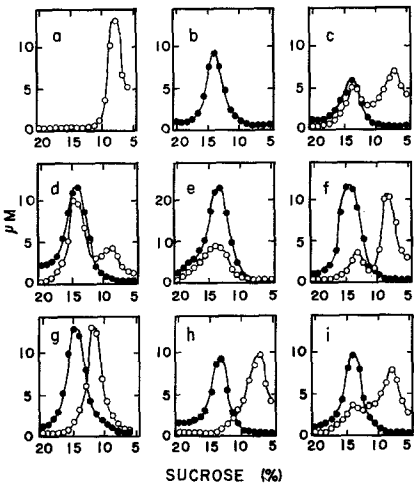


Fig. 3. Sucrose gradient profiles showing the distribution of adrenodoxin (-o-), spinach ferredoxin (-o-), adrenodoxin reductase (-o-), and P-450$_{scc}$ (-●-). (a) Fifteen nmoles of adrenodoxin, (b) 15 nmoles of P-450$_{scc}$, (c) 15 nmoles of adrenodoxin plus 7.5 nmoles of P-450$_{scc}$, (d) 15 nmoles of adrenodoxin plus 15 nmoles of P-450$_{scc}$, (e) 15 nmoles of adrenodoxin plus 30 nmoles of P-450$_{scc}$, (f) 15 nmoles of spinach ferredoxin plus 15 nmoles of P-450$_{scc}$, (g) 15 nmoles of adrenodoxin reductase plus 15 nmoles of P-450$_{scc}$, (h) 15 nmoles of adrenodoxin plus 14 nmoles of cholesterol-free P-450$_{scc}$, and (i) same as in h, except that the cholesterol-free P-450$_{scc}$ was treated with cholesterol before the centrifugation. P-450 was estimated by measuring the absorbance; $\varepsilon_{393} = 92 \times 10^3$ for cholesterol-bound form, and $\varepsilon_{418} = 105 \times 10^3$ for cholesterol-free form. For other conditions, see MATERIALS AND METHODS.

doxin reductase, no complex was obtained between the latter two adrenal steroid hydroxylase components (Fig. 3, g).

Our P-450$_{scc}$ preparation was obtained in a P-450$_{scc}$-substrate complex form containing a nearly stoichiometric amount of bound cholesterol (6). Therefore, the effect of omitting cholesterol on the complex formation was preliminarily investigated by similar sucrose density gradient centrifugation techniques. Fig. 3, h shows that no adrenodoxin was associated with the peak of the P-450$_{scc}$ when the mixture of the cholesterol-freed P-450$_{scc}$ and adrenodoxin together was examined in the absence of cholesterol. In a parallel experiment, this particular cholesterol-free preparation was treated with an excess of cholesterol and the resultant partly cholesterol-bound P-450$_{scc}$, as indicated by the rise in absorbance at 393 nm (6), was examined under similar conditions as in Fig. 3, h. As expected, the cholesterol-treated P-450$_{scc}$ bound some adrenodoxin (Fig. 3, i) indicating that cholesterol might participate in the complex formation between P-450$_{scc}$ and adrenodoxin.

To examine further the stoichiometric complex formation of P-450$_{scc}$ and

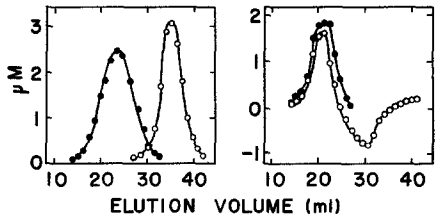


Fig. 4. Bio-gel P-300 column (0.9 x 72 cm) chromatogram of adrenodoxin and P-450$_{scc}$. (a) Adrenodoxin (-o-) or P-450$_{scc}$ (-●-), 22.5 nmoles of each was chromatographed on a separate column, and (b) 22.5 nmoles of P-450$_{scc}$ (-●-) in a similar buffer containing 1 μM adrenodoxin (-o-). For assay conditions, see the legend for Fig. 3.

adrenodoxin, gel filtration technique was employed. The elution profiles of P-450$_{scc}$ and adrenodoxin, each chromatographed separately on a Bio-gel P-300 column, are shown in Fig. 4, a. As can be seen in Fig. 4, b, when P-450$_{scc}$ was passed through a similar column in the presence of 1 μM adrenodoxin, a peak containing both P-450$_{scc}$ and adrenodoxin and a trough of the basal adrenodoxin were obtained.

In data not shown, the complex was fairly heat stable; heating for 20 min at 42° in 50 mM potassium phosphate buffer, pH 7.4, was not accompanied by loss in activity, but half of the activity was lost at 50°. In the absence of adrenodoxin, more than 90 % of the P-450$_{scc}$ was inactivated at 42° in 20 min. The spinach ferredoxin had some protective effect, while serum albumin was ineffective.

DISCUSSION

This report is the first conclusive demonstration of direct interaction between P-450$_{scc}$ and adrenodoxin, by spectral properties, density gradient ultracentrifugation, gel filtration and other properties. We thus conclude that P-450$_{scc}$, in the presence of cholseterol, combines with adrenodoxin to form a stable complex having a more rigid structure than adrenodoxin-free P-450$_{scc}$. The complex could be detected over a wide temperature range (5°-50°) and buffer concentration, either 10 mM or 50 mM.

It has been shown previously by Chu and Kimura (1) that adrenodoxin and adrenodoxin reductase form a 1:1 complex. The interaction between the two components has been extensively investigated by Hiwatashi et al. (2, 3) and by Lambeth et al. (4). On the basis of these and our evidence presented together, it is tempting to speculate that the three adrenal steroid hydroxyl-

ase components, in the presence of the substrate, may form an active 1:1:1 complex, which will be the subject of subsequent investigations.

It should be noted, in addition, that there have been numerous studies of the basis for changes in the spin state and ligand binding properties of cytochrome P-450 associated with the changes in the state of its surroundings. The ability of adrenodoxin to affect $P\text{-}450_{scc}$ as reported here suggests that the effect of adrenodoxin as well as that of cholesterol, the substrate, must be considered seriously as a factor in determining the properties of P-450, especially in various states of its existence either in the membrane structure or in the soluble preparation.

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