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Interaction of cytochrome P-450scc with cytochrome b_5

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Spectrophotometric, affinity chromatography and cross-linking experiments provided evidence that cytochrome P-450scc from bovine adrenocortical mitochondria forms a tight complex with cytochrome A_1 from rabbit liver microsomes. In the reconstituted system cholesterol side chain activity of cytochrome P-450scc was enhanced by the addition of cytochrome b_5.

Cytochrome P-450scc; Cytochrome b_5; Complex formation; Affinity chromatography; Chemical cross-linking

1. INTRODUCTION

Cytochrome P-450-dependent monooxygenases can be classified into two large groups [1]: (i) 'ferredoxin-dependent' monooxygenases each consisting of a flavoprotein, an iron-sulfur protein, and cytochrome P-450; (ii) 'microsomal' monooxygenases each containing only a flavoprotein and cytochrome P-450. Interrelation between these two groups of monooxygenases, regulation of the electron transfer in the membranes of endoplasmic reticulum and mitochondria, and interaction of the two groups of monooxygenases are unclear. Here we report that cytochrome P-450ssc from bovine adrenocortical mitochondria can form a tight complex with cytochrome b_5 from rabbit liver microsomes. Our results are consistent with the hypothesis that there is an interaction between 'microsomal' and 'ferredoxin-dependent' monooxygenases and that cytochrome b_5 may function as a mobile carrier between microsomes and mitochondria.

2. MATERIALS AND METHODS

2.1 *Chemicals*

Thyopropyl-Sepharose, Sepharose 4B, CNBr activated Sepharose 4B, Sephadex G-25 (fine) were from Pharmacia. Sodium cholate, cholesterol, pregnenolone, Tween 20, EDC from Serva.

2.2 *Purification of microsomal and mitochondrial enzymes*

Cytochrome P-450scc was purified from bovine adrenocortical mitochondria to electrophoretic homogeneity on immobilized adrenodoxin and cholate-Sepharose as described previously [2]. Intact cytochrome b_5 (d-b_5) containing the hydrophobic C-terminal sequence was purified as a by-product of purification of cytochrome P-450 LM_2 from phenobarbital treated rabbits [3].

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Abbreviation: EDC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide

2.3 *Immobilization of cytochrome P-450scc and cytochrome* b_5

Cytochrome P-450scc was immobilized on CNBr-activated Sepharose 4B in 100 mM phosphate buffer, pH 7.6; 70–80 nmol of cytochrome P-450scc were immobilized on each 1.0 ml of settled gel. Immobilization of cytochrome b_5 was performed in the same way, about 100 nmol of cytochrome b_5 were immobilized on 1 ml of settled gel.

2.4. *Spectrophotometric analysis of interaction of cytochrome P-450scc with cytochrome* b_5

Absorption spectra were recorded at room temperature in a Shimadzu UV-3000 or Specord M-40 spectrophotometer. The effect of cytochrome b_5 addition on the absorption spectrum of cytochrome P-450scc was measured by adding the same concentration of cytochrome b_5 in the reference cuvette to cancel the absorption due to cytochrome b_5.

2.5 *Cross-linking of cytochrome P-450scc cytochrome* b_5 *complex*

Cytochrome P-450scc and cytochrome b_5 were separately dialyzed against 10 mM phosphate buffer, pH 7.4. To a solution containing cytochrome P-450scc and cytochrome b_5 at a molar ratio of 1.3 in 10 mM phosphate buffer (pH 7.4) dry EDC was added to a final concentration of 2 mM and the mixture was allowed to stand for 30 min.

2.6 *Analytical methods*

SDS-PAGE was carried out according to Laemmli [4] in a Pharmacia GE-2/4 apparatus. All chromatographic steps were performed by using LKB equipment. Cytochrome P-450scc was determined by using a difference extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ (450–490 nm) for carbonmonoxyferrous cytochrome P-450 [5]. Cytochrome b_5 was estimated from the absolute spectrum of the oxidized hemeprotein using an extinction coefficient of 117 mM^{-1} cm^{-1} at 413 nm [6]. Cholesterol side chain cleavage activity of cytochrome P-450scc was determined in the reconstituted system in the presence of adrenodoxin reductase and adrenodoxin with and without cytochrome d-b_5 as described previously [2].

3. RESULTS

3.1. *Spectral evidence for cytochrome P-450scc interaction with cytochrome* b_5

The addition of cytochrome b_5 to cytochrome P-450scc in the presence of Tween 20 caused a partial shift of cytochrome P-450scc Soret peak from 417 to

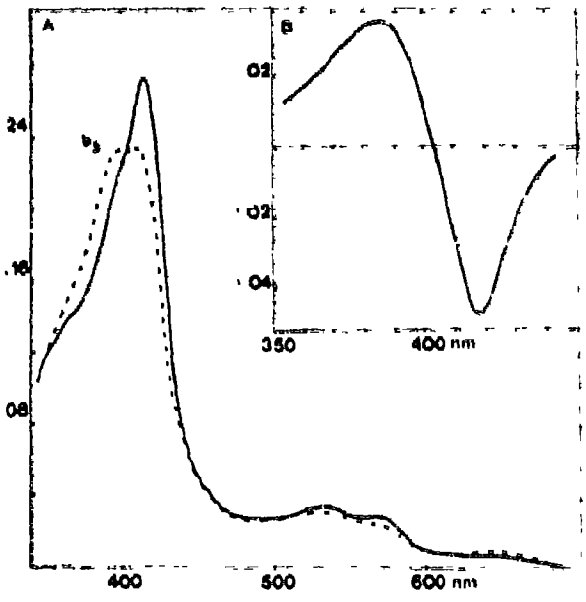


Fig. 1. Complex formation between cytochrome P-450scc and cytochrome b_5 as revealed by spectral analysis A, absolute spectra of Tween 20 induced low spin form of cytochrome P-450scc and its complex with cytochrome b_5, B, difference spectrum of the complex of cytochromes P-450scc and b_5 Spectra were recorded after addition of cytochrome b_5 (3 6 μM) to cytochrome P 450scc (4 2 μM) in 20 mM phosphate buffer containing 0 05% Tween 20

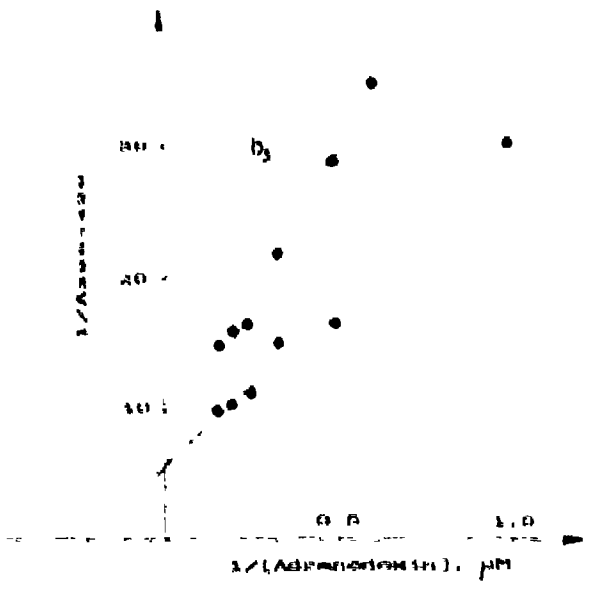


Fig. 2. Effect of cytochrome b_5 on the dependence of the spectral changes on adrenodoxin concentration. Cytochrome b_5 (2.4 μM) was added to cytochrome P-450scc (5 6 μM) in 20 mM phosphate buffer

393 nm, representing typical Type I spectral change (Fig. 1). By titrating cytochrome P-450scc with cytochrome b_5, a K_d value of 0.285 μM and an A_{max} value of 0.18 were determined. These values are close to those determined for cytochrome P-450scc interaction with adrenodoxin [7], indicating that both cytochrome b_5 and adrenodoxin interact with cytochrome P-450scc with high affinity.

3.2. *Competition of cytochrome* b_5 *with adrenodoxin for binding to cytochrome P-450scc*

To elucidate whether adrenodoxin and cytochrome b_5 bind to the same (overlapping) or different site(s) on the surface of cytochrome P-450scc molecule, spectral titration of cytochrome P-450scc was carried out with adrenodoxin in the presence of cytochrome b_5. It was found that cytochrome b_5 inhibited cytochrome P-450scc interaction with adrenodoxin and the inhibition was competitive (Fig. 2). This gives evidence that cytochrome b_5 competes with adrenodoxin for the binding to the same or overlapping site(s) on the cytochrome P-450scc molecule.

3.3. *Affinity chromatography of cytochrome P-450scc on cytochrome* b_5*-Sepharose 4B*

Fig. 3 shows the elution profile of cytochrome P-450scc on cytochrome b_5-Sepharose 4B column. As can be seen, a large quantity of cytochrome P-450scc applied could be eluted only when 1 M NaCl plus 0.3% sodium cholate were included in the elution buffer, indicating that it was tightly bound by immobilized cytochrome b_5. Both high-spin (HS) and low-spin (LS) forms of cytochrome P-450scc were able to interact with immobilized cytochrome b_5:

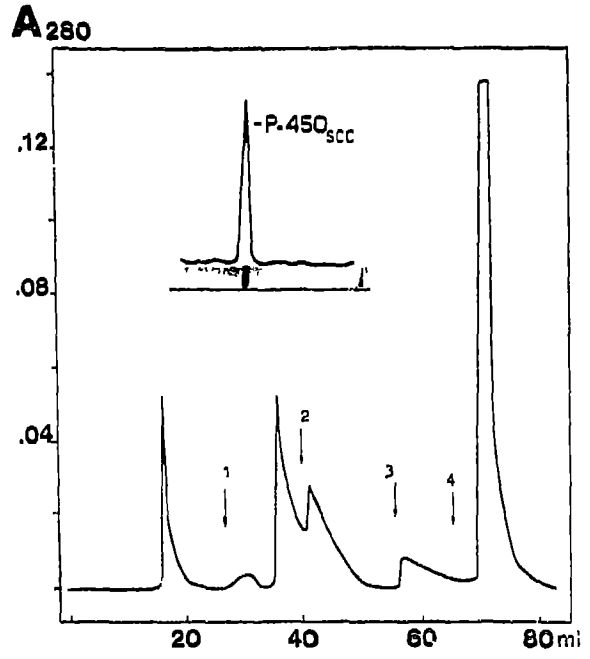


Fig 3 Affinity chromatography of partially purified cytochrome P-450scc on cytochrome b_5-Sepharose column (1 × 3 cm) Column was washed with equilibrating buffer [illegible] containing 0 1 M NaCl (1), 0 2 M NaCl (2), 0 5 M NaCl (3) and 1 0 M NaCl/0 3% sodium cholate (4) Inset SDS-PAGE of cytochrome P-450scc purified by affinity chromatography on cytochrome b_5-Sepharose.

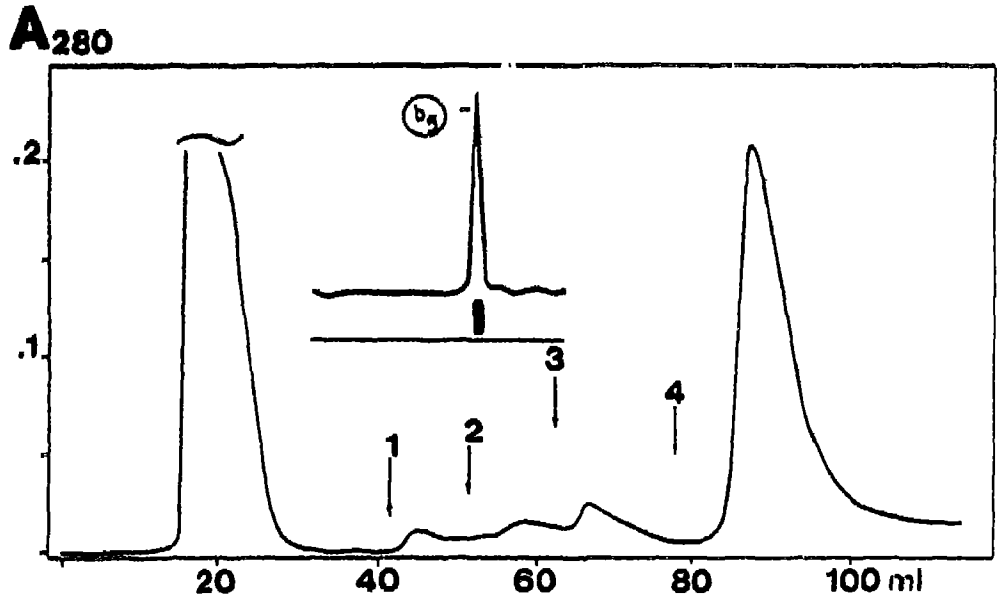
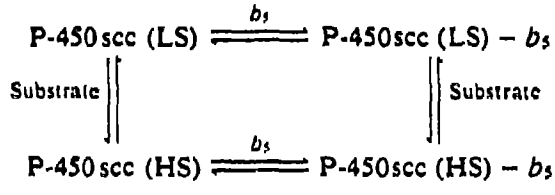


Fig. 4 Affinity chromatography of partially purified cytochrome b_5 on cytochrome P-450scc-Sepharose. Fractions of partially purified cytochrome b_5 containing both d-b_5 and t-b_5 were applied on cytochrome P-450scc-Sepharose (1 × 5 cm) Column was washed with the same buffer but containing 0.1 M NaCl/0 03% sodium cholate (1), 0 2 M NaCl/0 06% sodium cholate (2), 0 5 M NaCl/0 15 M sodium cholate (3), 1.0 M NaCl/0.3% sodium cholate (4) Inset: SDS-PAGE of cytochrome d-b_5 eluted from cytochrome P-450scc-Sepharose 4B column with 1.0 M NaCl/0.3% sodium cholate.



Rat and mouse cytochrome b_5 when immobilized are also a functional affinity columns for purification of cytochrome P-450scc (unpublished results).

3.4. *Affinity chromatography of cytochrome* b_5 *on cytochrome P-450scc-Sepharose 4B*

Affinity chromatography of cytochrome b_5 on cytochrome P-450scc-Sepharose column (Fig. 4) allowed us to conclude that both native cytochrome b_5 (d-b_5) and its hydrophilic fragment (t-b_5) which was also present in the starting material interact with immobilized cytochrome P-450scc. While cytochrome t-b_5 could be eluted by increasing ionic strength, cytochrome d-b_5 bound to affinity column much more strongly and could be eluted only when 1 M NaCl plus 0.3% sodium cholate were included in the elution buffer (Fig. 4, inset). Thus, both hydrophilic and hydrophobic domains of cytochrome b_5 seem to be involved in the interaction with cytochrome P-450scc, and both electrostatic and hydrophobic interactions contribute to the complex formation. An interesting finding was that the binding capacity of the cytochrome P-450scc-Sepharose decreased upon multiple chromatography runs. This decrease was found to be due to the loss of heme from immobilized cytochrome P-450scc. Complete removal of heme with chaotropic reagents resulted in total loss of the binding capacity of the column. This indicates that, in addition to electrostatic and hydrophobic interactions, the heme in cytochrome P-450scc is an important prerequisite for an effective interaction.

3.5. *Chemical cross-linking of cytochrome P-450scc with cytochrome* b_5

The water-soluble cross-linking reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was used to study the interaction of cytochrome P-450scc with cytochrome b_5. Fig. 5 shows the SDS-PAGE patterns of a mixture of cytochrome P-450scc and cytochrome b_5 before and after treatment with EDC. After the treatment, a new band appeared, the molecular weight of which corresponded to the sum of the molecular weights of both hemeproteins.

3.6. *Stimulation of cytochrome P-450scc catalyzed reaction by cytochrome* b_5

The cytochrome P-450scc-catalyzed conversion of cholesterol to pregnenolone in the reconstituted system in the presence of adrenodoxin reductase and adrenodoxin was stimulated by 75% and 100% when cytochrome d-b_5 was added to the incubation mixture at b_5:P-450 molar ratios of 2:1 and 5:1, respectively. Cytochrome b_5 could not however substitute adrenodoxin in cytochrome P-450scc-catalyzed conversion of cholesterol to pregnenolone in the reconstituted system despite of the fact that adrenodoxin reductase

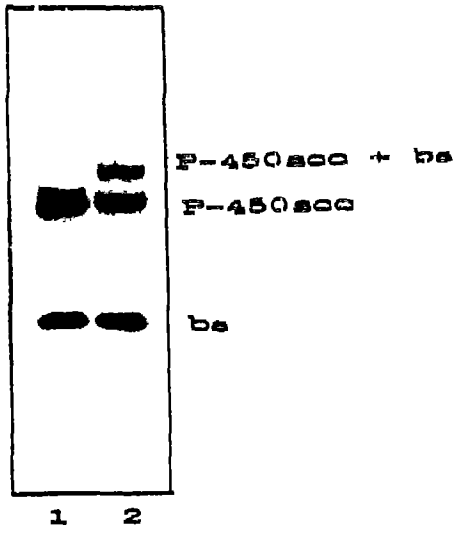


Fig. 5 SDS PAGE of EDC catalyzed cross-linking of cytochrome b_5 with cytochrome P-450scc. Gel 1, cytochrome P-450scc and cytochrome b_5; gel 2, cross-linked complex

can reduce cytochrome b_5 at one order less rate than adrenodoxin (not shown).

4. DISCUSSION

In the present work we have demonstrated that cytochrome b_5 binds tightly to cytochrome P-450scc. This finding broadens the functional role of cytochrome b_5 in the processes of electron transfer not only in 'microsomal' cytochrome P-450-dependent monooxygenases but also in 'ferredoxin-dependent' cytochrome P-450-catalyzed reactions and indicate a common recognition mechanism of a highly specific protein–protein interaction. The physiological relevance of the complex of cytochrome P-450scc with cytochrome b_5 is still unknown. The association of cytochrome o_5 with cytochrome P-450scc involves at least two binding mechanisms: complementary charge ion pairing and hydrophobic interaction of corresponding hydrophobic surfaces. The failure of apocytochrome P-450scc to form a complex with cytochrome b_5 indicates that heme of cytochrome P-450scc is also an important prerequisite for the functional interaction. These results suggest the involvement of specific carboxylate residues of cytochrome b_5 and adrenodoxin as well as the heme pocket of cytochrome b_5 in complex formation with cytochrome P-450scc and indicate that both proteins contain special recognition sites on the surface for electrostatic interaction which have also an intrinsic property to discriminate electron-transfer partners. The competition of cytochrome b_5 and adrenodoxin for binding with cytochrome P-450scc suggests that a similar cationic site on the surface of cytochrome P-450scc is involved in the association with both proteins. Indeed, recently we have shown by chemical modification and cross-linking experiments that positively charged residues of the N- and C-terminal sequence of cytochrome P-450scc are involved in the electrostatic interaction with adrenodoxin [8,9].

The similarity of the processes of the interaction of cytochrome P-450scc with cytochrome b_5 and adrenodoxin allowed us to propose that adrenodoxin might interact with cytochrome P-450 from endoplasmic reticulum membranes. Indeed, the interaction of adrenodoxin with some microsomal cytochrome P-450 isozymes has been demonstrated using spectral titration, affinity chromatography and reconstitution experiments (unpublished results). Moreover, interaction of bacterial ferredoxins (putidaredoxin and linoredoxin) and their ability to transfer electrons to cytochrome P-450 LM_2 was also shown [10]. Finally, similarity between cytochrome b_5 and adrenodoxin does not exclude the possibility of electron transfer to ferredoxin from NADH-cytochrome b_5 reductase, that may indicate new interrelations between NADPH- and NADH-dependent electron transfer systems.

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