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Expression of Cytochrome P450scc in *Escherichia coli* Cells: Intracellular Location and Interaction with Bacterial Redox Proteins

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Abstract—*Escherichia coli* cells producing the mature form of adrenal cytochrome P450scc were used as a model for study of cytochrome P450scc topogenesis. By disruption of transformed *E. coli* cells and centrifugation of the homogenate under conventional conditions, we obtained membrane and soluble (high-speed supernatant) fractions both containing the recombinant protein. Gel-permeation high performance liquid chromatography showed that in the high-speed supernatant the native cytochrome P450scc exists exclusively as a component of membrane fragments exceeding 400 kD. These data supported by kinetic assays suggest that the >400-kD particles containing P450scc are lipoprotein associates. In total, we failed to detect a genuine soluble cytochrome P450scc in the *E. coli* cells, which suggests that membrane insertion is an obligatory stage of holoenzyme formation. In the high-speed supernatant supplemented with NADPH, cytochrome P450scc underwent one-electron reduction and could convert 22R-hydroxycholesterol into pregnenolone. Thus, we have for the first time observed functional coupling of cytochrome P450scc with the bacterial electron transfer system.

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Cytochrome P450scc (CYP11A1) is a component of the side-chain cleavage system of adrenocortical mitochondria that catalyzes conversion of cholesterol into pregnenolone, a precursor to steroid hormones. In adrenocortical cells, P450scc is synthesized as a precursor bearing an N-terminal cleavable sequence [1, 2], presumably on free polysomes; the precursor is imported into mitochondria and undergoes proteolytic processing, whereby the mature form is incorporated into the inner membrane [3].

P450scc is quite an unusual protein. It has no segments rich in nonpolar residues and long enough to serve as transmembrane domains [4, 5]. However, inasmuch as P450scc is not released from the inner mitochondrial membrane upon carbonate treatment [6], it can be classed with integral membrane proteins. Moreover, the data on the accessibility of selected epitopes to antibodies in mitoplasts and inside-out submitochondrial particles suggested a model [7] according to which P450scc is composed of two domains, each spanning the inner mitochondrial membrane. Experiments with various inhibitors and electron acceptors differing in their ability to penetrate the lipid bilayer indicated that the P450scc substrate-binding site faces the mitochondrial matrix [8].

Later, analysis of rotational diffusion of P450scc in proteoliposomes [9] and freeze-fracture electron microscopy of such vesicles [10] led to the conclusion that this protein is integrated into the lipid bilayer without traversing it. Although the proteoliposome model gives some insight into the character of P450scc interaction with phospholipid membranes, it is poorly informative as regards the topogenesis of this protein. In fact, these model experiments considered the interaction between liposomal membranes and the mature holoenzyme, which hardly takes place in nature. On the contrary,

Abbreviations: Adx) adrenodoxin; AdR) adrenodoxin reductase; CYP11A1) cytochrome P450scc; DTT) dithiothreitol; IMV) inverted membrane vesicles; SMP) submitochondrial particles.

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according to present-day notions, in most cases the import of proteins into the inner mitochondrial membrane involves translocation of unfolded polypeptide chains followed by protein folding/heme incorporation/membrane insertion [11-13].

Somewhat closer to reality are experiments based on expression of cytochrome P450scc in *E. coli* cells. Wada et al. [14] were the first to show that in this case a newly synthesized mature form of CYP11A1 undergoes correct folding and binds the heme, thus forming the catalytically active holoenzyme incorporated into the plasma membrane of bacterial cells. Recent investigations of the P450scc membrane topology based on *E. coli* cells model by site-directed mutagenesis and fluorescence microscopy [15] have shown that the P450scc molecule reaches the membrane bilayer interface by the F-G loop located in the middle region of the polypeptide chain. In line with this, mutations in the F-G loop raised the amount of the recombinant proteins in the soluble fraction of *E. coli* cells [16]. Besides, binding of the P450scc molecule with the *E. coli* plasma membrane probably involves its N-terminal A′ helix. Removal of this fragment reduced the P450scc affinity to the membrane, which manifested itself as increased content of the protein in the soluble fraction of bacterial cells [15]. In these publications [15, 16], P450scc produced in *E. coli* cells could be detected both in a "membrane" and "soluble" subcellular fractions after high-speed centrifugation of the homogenate prepared from sonicated cells. However, no data have been produced about the real physical state of P450scc in the "soluble" fraction.

More detailed studies on the properties and intracellular location of P450scc produced in *E. coli* might shed light on the order and mutual interrelation of individual stages of topogenesis of this protein. Obviously, the following questions should be answered first of all: (i) whether insertion of the CYP11A1 polypeptide into the plasma membrane proceeds cotranslationally or posttranslationally, (ii) whether heme binding precedes or follows the polypeptide insertion into the membrane, and (iii) whether membrane insertion is an indispensable condition for proper folding of the CYP11A1 chain.

The goal of this work was to ascertain the intracellular location of P450scc synthesized in the *E. coli* cells, with the main purpose of clarifying its physical state in the "soluble" fraction.

MATERIALS AND METHODS

Cells, plasmids, and media. We used *E. coli* strain JM109 (Promega, USA) and the pTrc99A vector with the hybrid *trp/lac/trc* promoter [17]. The pTrc99A/mCYP11A1 plasmid containing the nucleotide sequence for the mature form of cytochrome P450scc from bovine adrenal cortex was a generous gift of Dr. M. R. Waterman (Vanderbilt University, USA) [14]. The bacterial cultivation media were from Difco (USA).

Expression of P450scc in *E. coli*. Expression of the mature form of CYP11A1 in *E. coli* was performed as described earlier [14]. The competent bacterial cells were transformed with the recombinant plasmid according to a standard procedure [18]. Gene expression was initiated by adding isopropyl-1-thio-β-D-galactopyranoside (0.5 mM) into the growth medium supplemented with δ-aminolevulinic acid (0.5 mM). The culture was then incubated for 40 h at 24°C with vigorous shaking (140 rpm).

Cell fractionation. Bacterial cells (1 liter of culture of $A_{600} \approx 6$-7) were harvested by centrifugation (10 min at 7500*g*), washed with buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $MgSO_4$), centrifuged for 10 min at 7500*g*, and suspended in 20 mM sodium phosphate, pH 7.4, 5 mM $MgSO_4$, 0.2 mM phenylmethylsulfonyl fluoride, 4 mM dithiothreitol (DTT). The cells were disrupted with a French press (maximum pressure of 16,000 psi). The resulting homogenate was centrifuged for 15 min at 14,000*g* to remove cell debris and inclusion bodies that could have formed upon synthesis of the recombinant protein. The clarified homogenate was centrifuged for 1 h at 120,000*g*. The pellet was suspended in 50 mM sodium phosphate buffer (pH 7.4) and considered to be the fraction of inverted membrane vesicles (IMV). This material and the supernatant were mixed with glycerol (final concentration 20%) and stored at –20°C. To prepare spheroplasts, the cells were sedimented at 3000*g* (5 min), suspended in 10 mM Tris-HCl (pH 8.0) with 5 mM EDTA, incubated for 10 min at room temperature, centrifuged again (3000*g*, 5 min), and suspended in 10 mM Tris-HCl (pH 8.0) with 0.25 M sucrose and 5 mM EDTA. Then lysozyme was added (0.15 mg/ml), and spheroplast formation was monitored with a microscope. The process was stopped by adding $MgCl_2$ to 100 mM.

Spectral analysis. Cytochrome P450scc concentration in subcellular fractions was determined from CO-difference spectra of the preparations reduced with dithionite, using extinction coefficients ($mM^{-1}\cdot cm^{-1}$) $\varepsilon_{450\text{-}490} = 91$ for the native and $\varepsilon_{420\text{-}490} = 110$ for the denatured form [19]. The concentrations of adrenodoxin (Adx) and adrenodoxin reductase (AdR) were estimated from absolute absorption spectra of oxidized samples using $\varepsilon_{414} = 9.8$ and $\varepsilon_{455} = 11$ $mM^{-1}\cdot cm^{-1}$, respectively [20].

High performance liquid chromatography (HPLC). For this purpose we used a Shimadzu LC-10AD chromatograph (Shimadzu, Japan) equipped with an SPD-M10A UV/VIS photodiode detector. Gel-permeation chromatography was performed in a 0.8 × 63 cm Toyopearl-60 column (Toyo Soda, Japan). The void volume of the column was estimated using Blue Dextran ($2\cdot10^6$ daltons). Ferritin, catalase, bovine serum albumin, and cytochrome *c* (molecular masses 400, 220, 67, and 12.5 kD, respectively) were used for column calibration. The following buffers were applied: buffer A containing

50 mM sodium phosphate (pH 7.2), 0.1 mM EDTA, and 0.1 mM DTT; buffer A with 0.3% Emulgen 913 (AE); buffer A plus Emulgen 913 plus 0.5 M NaCl (AES). The elution rate was 0.5 ml/min with a sample volume of 200 μl. Absorption spectra were taken in the 350-700 nm range. Chromatographic data were analyzed using Shimadzu CLASS VP software (Japan). P450scc was identified by conversion of [^{3}H]cholesterol (10 μM) into pregnenolone in a system containing aliquots of eluted protein (100 μl), Adx (1 nmol), and AdR (0.2 nmol). The reaction was started by adding NADPH (200 μM) and performed in 1 ml of buffer AE at 25°C for 30 min. The extent of conversion was estimated after separating the steroids by thin layer chromatography (TLC) [21].

Affinity chromatography on Adx-Sepharose. This procedure was performed using an automatic GradiFrac system (Pharmacia Biotech, USA) in two alternative ways. First, 10 ml of high-speed supernatant containing 5 nmol of P450scc was dialyzed against buffer A (see above) and loaded on an Adx-Sepharose column. The column was then washed with buffer A; the same buffer with 1 M NaCl and 0.3% sodium cholate was used for elution [21]. Second, 20 ml of high-speed supernatant was initially solubilized with sodium cholate (final concentration 0.5%) and fractionated with ammonium sulfate (27-43% saturation). The final precipitate was dissolved in buffer A containing 0.03% sodium cholate and passed through the Adx-Sepharose column as above.

Spectrophotometric titration with steroids and adrenodoxin. Use was made of a Perkin Elmer Lambda 40 spectrophotometer (Perkin Elmer, USA) with tandem cuvettes. Formation of P450scc complexes with cholesterol and Adx was detected by the type I spectral changes (390 against 422 nm) upon spectrophotometric titration [22, 23]. To determine the K_D for the Adx–P450scc complex, the fractions of free and bound Adx were estimated. The accessibility of substrate- and Adx-binding sites in various P450scc preparations was estimated from the data of spectrophotometric titration using $\varepsilon_{390\text{-}420}$ = 115 $mM^{-1}\cdot cm^{-1}$ for 100% conversion of P450scc from low-spin to high-spin form [22, 23].

Enzymic reduction of P450scc. This process was followed under anaerobic conditions (in the presence of D-glucose, glucose oxidase, and catalase) in buffer A saturated with carbon monoxide. Cytochrome P450scc concentration in the samples was 0.25 μM. The reaction mixture was supplemented with Adx (0.05 μM) and AdR (0.01 μM). The reaction was started by adding NADPH (final concentration 50 μM). The increase in absorbance $\Delta_{450\text{-}490}$ was registered with a Shimadzu UV-1202 spectrophotometer. The rate constant (k_1) of the CO complex formation was calculated using a program for kinetic measurements. Upon reaching the steady-state $\Delta_{450\text{-}490}$, the CO difference spectrum was registered. The degree of enzymic reduction was calculated relative to chemical reduction with dithionite taken as 100%.

Sterol side-chain cleavage. The reaction was performed with 1 ml of the high-speed supernatant containing 0.25 nmol of cytochrome P450scc or with the same amount of isolated cytochrome P450scc in buffer AE (see HPLC). The reaction mixture contained 50 μM cholesterol, 0.4 μCi [^{3}H]cholesterol, 0.25 nmol AdR, and 2.5 nmol Adx. After 10-min preincubation at 25°C, the reaction was started by adding NADPH (final concentration 0.25 mM). The samples were incubated in a thermostatted shaker at 25°C for 10-30 min. Steroids were extracted with ethyl acetate (twice with 2.5 ml). The extracts were evaporated and analyzed by TLC using silica gel plates and a solvent system comprising hexane, diethyl ether, and acetic acid at volume ratios of 15 : 15 : 1, respectively. Zones containing steroids were revealed with $FeCl_3$ dissolved in acidic *n*-butanol, cut out, and analyzed with a Mark III scintillation counter (Tracor Analytic, USA). Reaction products in the enzymic system were also detected by HPLC after conversion of 22R-hydroxycholesterol into pregnenolone and progesterone after addition of cholesterol oxidase (0.45 unit, 45 min incubation at 37°C) [24]. The mixture was applied to a 4.6 × 250 mm LiChrosorb RP-18.5 μm column, and eluted in a gradient of acetonitrile in water, 20-100% over 15 min.

Other methods. Protein content was measured after Lowry et al. [25]. Cytochrome P450scc was visualized by Western blotting [26] after SDS-PAGE (10% acrylamide) [27] using the IgG fraction of the bovine anti-P450scc antiserum. Trypsin treatment of preparations was as follows. Aliquots of IMV and spheroplasts (final concentration 1 mg/ml) were suspended in 50 mM HEPES (pH 7.2), 0.6 M sorbitol, 80 mM KCl, and incubated with trypsin (50 μg/ml) for 30 min on ice. Trypsin was then inactivated with soybean inhibitor (0.8 mg/ml). Succinate dehydrogenase activity was assayed as in [28]. Submitochondrial particles (SMP) were prepared from bovine adrenocortical mitochondria by sonication [20] and solubilized for gel-permeation chromatography in buffer AES. Bovine P450scc, Adx, and AdR were prepared by published protocols [20, 29, 30]. Reconstitution of the cholesterol monooxygenase/lyase system was carried out using a bovine cytochrome P450scc preparation purified by chromatography on a cholate-Sepharose column [31]. The spectrophotometric purity index for this preparation was A_{393}/A_{280} = 0.93. Statistical data processing was carried out with the use of Microsoft Excel 2000.

RESULTS AND DISCUSSION

Recovery of P450scc in *E. coli* cell fractions. According to the data of SDS-PAGE and immunoblotting with anti-P450scc antibodies, both the membranes and the supernatant derived from transformed bacterial cells contained a protein of molecular mass corresponding to that of the bovine cytochrome P450scc (53 kD).

The CO difference spectra showed that the content of native cytochrome P450scc in the membranes obtained from 1 liter of culture was 12.5 ± 3.0 nmol (plus 4.4 ± 1.5 nmol of denatured form); the respective supernatant contents were 18.7 ± 4.0 and 2.8 ± 1.0 nmol. Thus, in both fractions cytochrome P450scc was largely in the native form in commensurate amounts.

Arrangement of P450scc in the *E. coli* plasma membrane. In adrenocortical mitochondria, P450scc is integrated into the inner membrane in such a way that its unique trypsin-sensitive site faces the matrix. Accordingly, trypsinolysis of inside-out SMP preparations yields two P450scc fragments of 26 and 29 kD [7, 32]. To ascertain the arrangement of recombinant P450scc in the bacterial plasma membrane, we exposed spheroplasts and IMV prepared from the *E. coli* cells to trypsin. No P450scc fragments were produced in spheroplasts (Fig. 1, lane *1*). In the IMV preparations, the trypsin-sensitive site of the recombinant protein was accessible to trypsin, as evidenced by the accumulation of its 26-, 29-, and 40-kD fragments (Fig. 1, lane *2*).

It has earlier been shown that tryptic cleavage of denatured cytochrome P450scc produces a 40-kD fragment in addition to those obtained with the native protein [30]. Later, a 40-kD fragment has been observed [6] upon trypsin treatment of SMP prepared from adrenocortical mitochondria with pre-P450scc imported *in vitro*, and supposed to arise from incompletely folded molecules. Thus, our data signify that cytochrome P450scc in the bacterial plasma membrane is oriented in the same way as in the inner membrane of adrenocortical mitochondria. The additional 40-kD tryptic product results either from some misfolding of the recombinant protein or from partial denaturation during the isolation procedure.

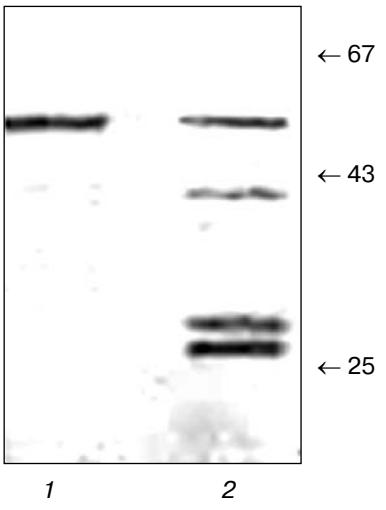

Fig. 1. Arrangement of cytochrome P450scc in the *E. coli* plasma membrane. Spheroplasts and IMV prepared from the *E. coli* cells transformed with the pTrc99A/mCYP11A1 plasmid were subjected to SDS-PAGE followed by immunoblotting after trypsinolysis. An IgG fraction of antiserum against bovine cytochrome P450scc was used for immunoblotting. Lanes: *1*) spheroplasts after trypsinolysis; *2*) IMV after trypsinolysis. Arrows show location of marker proteins in the gel.

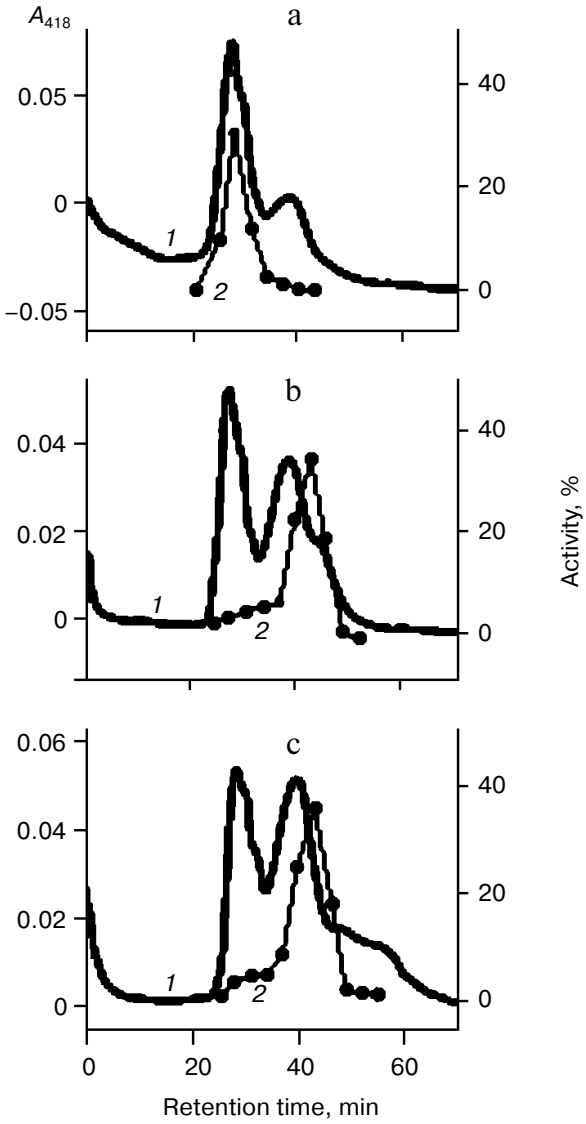

Fig. 2. Size distribution and some properties of *E. coli* plasma membrane fragments in the high-speed supernatant (homogenate centrifugation at 120,000*g* for 1 h). Gel-permeation HPLC profiles obtained with various eluting buffer systems. a) Buffer A: 0.05 M sodium phosphate (pH 7.2) with 0.1 mM EDTA and 0.1 mM DTT; b) buffer A plus Emulgen 913 (0.3%); c) buffer A plus Emulgen 913 (0.3%) and 0.5 M NaCl. Curves: *1*) absorbance of eluates at 418 nm (A_{418}); *2*) the levels (% of maximum) of conversion of cholesterol into pregnenolone (activity) after addition of Adx, AdR, and NADPH into the eluate fractions (see details in "Materials and Methods").

State of supernatant P450scc. The high-speed supernatant was concentrated about fivefold in Amicon test tubes (Millipore, USA). The resulting sample was subjected to gel-permeation HPLC. In Fig. 2 the elution profiles are shown (curves *1*) as absorbance at 418 nm.

When needed, absorption spectra were registered in the 350-700 nm range. When buffer without detergent (buffer A) was used for elution, heme-containing material was released in the void volume of the column (Fig. 2a, curve *1*). The ability of converting cholesterol into pregnenolone in the presence of added Adx and AdR was detected only in the fraction of particles exceeding 400 kD (Fig. 2a, curve *2*). Thus, no monomeric P450scc is found in the high-speed supernatant. On the other hand, SDS-PAGE revealed that the >400-kD fraction contains a set of 15-60-kD proteins, suggesting that P450scc and accompanying proteins in the supernatant are in the form of protein aggregates or lipoprotein particles.

HPLC in the presence of Emulgen 913 resulted in a higher peak at 37.8 min corresponding to 180-kD particles (Fig. 2b, curve *1*). Under the same conditions, the P450scc activity peaked at 42 min, corresponding to 115-kD particles (Fig. 2b, curve *2*). As it was shown in [33], P450scc molecules tend to aggregate as a result of hydrophobic interrelations and the micellar structures with apparent molecular weight 115 kD arise when the amount of nonionic detergents is more than critical. In our experiment the addition of Emulgen 913 probably led to solubilization of heterogeneous protein complexes and P450scc homo-oligomers are formed.

Attenuation of electrostatic interactions by adding salt to the AE buffer did not change the P450scc retention time (Fig. 2c, curve *2*). However, the chromatogram featured proteins of 10-60 kD (retention times of 45-60 min); it followed from absolute visible spectra that this fraction contained hemo- and flavoproteins (not shown). Comparing the elution profiles of the high-speed supernatant (Fig. 2c) and solubilized membranes of *E. coli* (Fig. 3a), one can clearly see differences in their composition. On the other hand, the elution profile for the *E. coli* solubilized membranes closely paralleled that for adrenocortical SMP (Fig. 3, a and b) although in the latter case much more flavoproteins accompanied cytochrome P450scc (compare Figs. 3c and 3d). In particular, the absorption spectrum of the SMP protein fraction with retention time of 42 min (Fig. 3d) was a superposition of heme (maximum 420 nm) and flavin chromophores (maximum 455 nm, shoulder at 475 nm).

The HPLC data correlate with the results of succinate dehydrogenase assay in the *E. coli* high-speed supernatant. Succinate dehydrogenase can be considered a genuine marker enzyme of the bacterial plasma membrane. In our hands, the overall succinate dehydrogenase activities of membranes and supernatant related as 55 and 45%. This agrees closely with the data on the P450scc content in these fractions. Upon heavier centrifugation of the same homogenate (120,000*g* for 2.5 h), the portions of succinate dehydrogenase activity in the pellet and supernatant proved to be 85 and 15%, respectively. For comparison, the P450scc contents in these fractions related as 87 and 13%. On the strength of these data, one may conclude that under the conditions of bacterial cell homogenate fractionation described in the literature (see, e.g. [16]) the so-called "soluble fraction" certainly contains plasma membrane fragments.

Adx-Sepharose does not bind P450scc in the high-speed supernatant. If at least some of the above high-molecular-weight associates were native oligomers, they could be isolated by affinity chromatography on immobilized Adx. This is a routine for isolating cytochrome P450scc from adrenocortical mitochondria [29, 30]. However, the Adx-Sepharose column retained only a negligible amount of protein with CO-difference spectra characteristic of cytochrome P450scc. The bulk of hemoprotein was freely eluted with other proteins in the volume of the loaded sample. Addition of cholesterol (10 μM) to convert P450scc into the high-spin form did not improve affinity binding. It was only pretreatment of the supernatant with 0.5% sodium cholate in the presence of cholesterol followed by fractionation with ammonium sulfate (protein delipidation and separation) that allowed cytochrome P450scc to be isolated by affinity chromatography. Even in this case, the yield did not exceed 20%. These data once again suggest that the supernatant cytochrome P450scc is a component of lipoprotein particles in which it cannot be captured by Adx-Sepharose, though it can interact with free Adx (see below).

Functional properties of P450scc in *E. coli* subcellular fractions. The table summarizes some functional characteristics of P450scc, isolated from adrenocortical mitochondria, and of recombinant protein in membranes and supernatant of the *E. coli* cells. In the absence of nonionic detergents, isolated cytochrome P450scc exists mainly in the high-spin form, which is conditioned by binding of endogenous cholesterol (table, preparation 1). At first, the detergents were added to increase the portion of the low-spin form, disaggregate the protein, and enhance the solubility of its substrate. Restitution of the initial high-spin form induced by substrates and Adx testifies to P450scc complexing with its natural ligands. Cholesterol and Adx bind with P450scc to cause type I changes in the difference spectra (λ_{max} = 390 nm, λ_{min} = 422 nm). From the dependence of type I spectral changes on the concentration of the above ligands in double reciprocal plots, we determined the dissociation constants of the corresponding complexes and the accessibility of the P450scc substrate-binding site for cholesterol in preparations 1-4.

There are no significant differences in these characteristics for preparations 1-3 (table), which is indicative of similar accessibility of their P450scc substrate-binding centers to cholesterol. However, some difference between preparations 3 and 4 may be accounted for by a negative effect of Emulgen 913 on the cholesterol binding to P450scc, which was earlier observed for the isolated enzyme.

Cytochrome P450scc is obtained from *E. coli* cells in the low-spin form [14], which does not exhibit significant

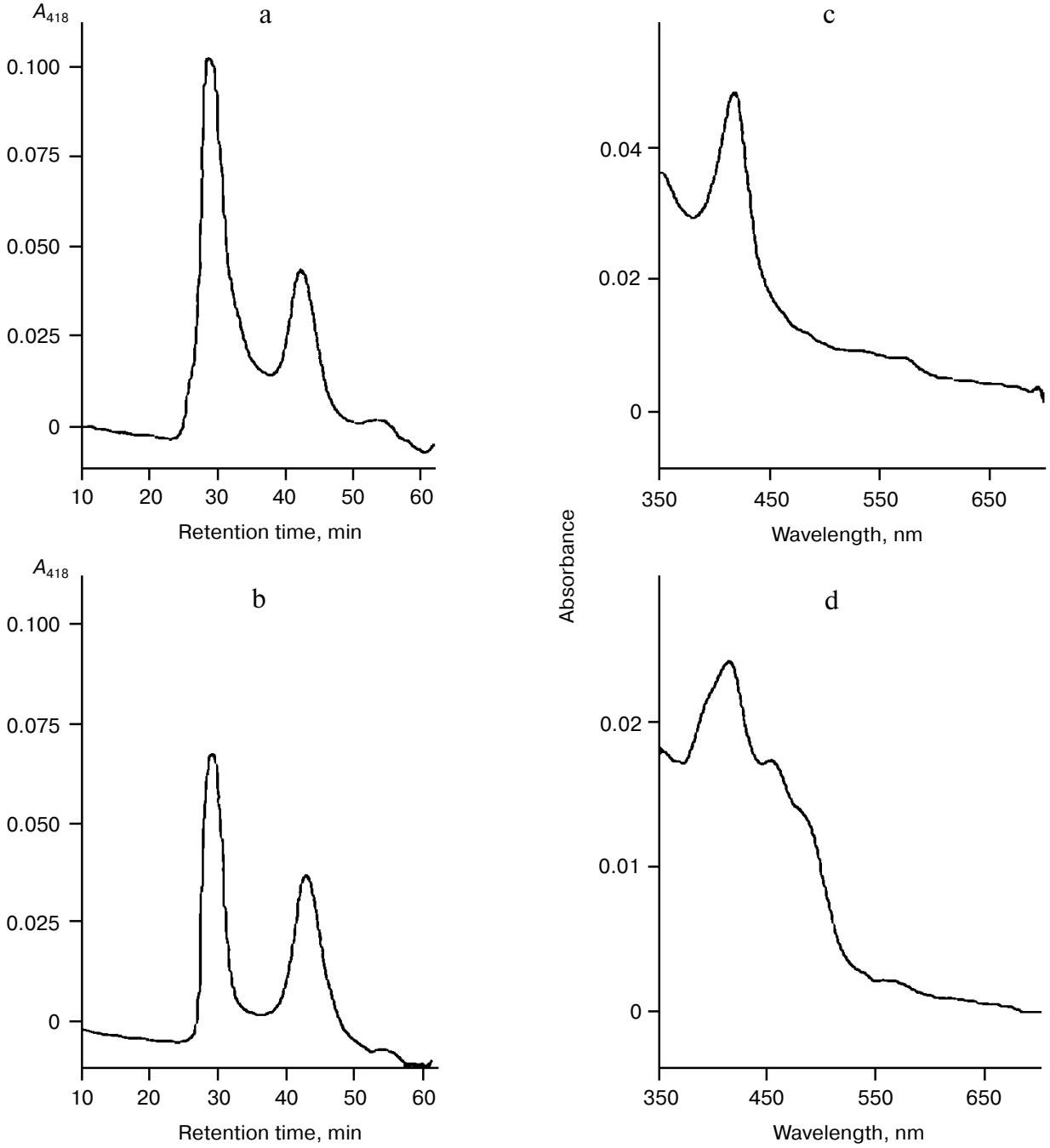

Fig. 3. Size distribution and spectral characteristics of fragments in solubilized *E. coli* membranes (pellet after centrifugation of cell homogenate at 120,000*g* for 1 h) and adrenocortical SMP. Gel-permeation HPLC profiles (50 mM sodium phosphate (pH 7.2), 0.1 mM EDTA, 0.1 mM DTT, 0.3% Emulgen 913, 0.5 M NaCl) of the *E. coli* membranes (a) and SMP (b). Panels (c) and (d) are the absorption spectra of the respective 42-min eluates.

spectral changes upon interaction with Adx. Therefore, to study Adx complexing with P450scc we supplemented preparations 1-4 with 25 μM cholesterol. The accessibility of the Adx-binding site was 80-95% (table), i.e., in all four preparations the Adx-binding sites of the cytochrome were almost completely exposed to the aqueous medium. The affinity of P450scc to Adx in preparations 2 and 3 was 4-5 times lower than that of the authentic bovine cytochrome; in this respect, the state of P450scc in the high-speed supernatant is similar to that in the membrane fraction and different from that of the isolated protein.

The supernatant P450scc undergoes one-electron reduction even in the absence of added Adx and AdR.

Functional characteristics of various cytochrome P450scc-containing preparations

Parameter	Preparations			
	1	2	3	4
Specific content, nmol/mg protein	18.0 ± 2.0	0.04 ± 0.02	0.02 ± 0.015	0.02 ± 0.017
K_D for cholesterol, μM (accessibility of substrate-binding center, %)	60.2 ± 15.2 (60.4 ± 10.3)	50.1 ± 10.1 (55.0 ± 5.4)	40.3 ± 4.5 (55.1 ± 6.5)	35.4 ± 5.6 (75.4 ± 4.8)
K_D for Adx, μM (accessibility of Adx-binding center, %)	0.12 ± 0.02 (90.1 ± 4.5)	0.45 ± 0.15 (95.3 ± 4.0)	0.50 ± 0.05 (80.2 ± 6.1)	0.80 ± 0.1 (85.1 ± 6.3)
Enzymic reduction level, %				
+ NADPH	*	*	5.2 ± 2.0	80.4 ± 4.4
+ NADPH + AdR + Adx	90.3 ± 3.4	80.2 ± 6.1	85.0 ± 4.4	85.1 ± 5.3
Turnover in 22R-hydroxycholesterol transformation, min^{-1}				
+ NADPH	*	*	0.5 ± 0.2	2.6 ± 0.5
+ NADPH + AdR + Adx	26.1 ± 3.2	30.1 ± 4.2	32.2 ± 1.5	27.0 ± 2.7
Turnover in cholesterol transformation, min^{-1}				
+ NADPH	*	*	0.09 ± 0.03	0.35 ± 0.15
+ NADPH + AdR + Adx	3.8 ± 0.5	2.9 ± 0.6	3.0 ± 0.3	3.3 ± 0.3

Note: Preparations: 1) purified adrenocortical cytochrome P450scc (plus Emulgen, 0.3%); 2) high-speed pellet from the *E. coli* homogenate (plus Emulgen, 0.3%); 3) high-speed supernatant from *E. coli* homogenate (plus Emulgen, 0.3%); 4) same as 3 without detergent. The *E. coli* fractions were separated by centrifugation of the cell homogenate (12,000*g*, 1 h). The values (mean ± SD) from at least three experiments are given.

* No effect.

Indeed, addition of NADPH to this fraction results in a CO difference spectrum typical of P450scc (Fig. 4). In preparation 4 (no detergent) (Fig. 4, curve *1*), such enzymic reduction reaches 80% of the chemical reduction with dithionite; but it drastically decreases in the presence of Emulgen 913 (preparation 3) (Fig. 4, curve *2*). These data testify to the interaction of recombinant P450scc with bacterial redox protein(s) capable of transferring electrons from NADPH to this foreign protein. Moreover, the inhibitory effect of the detergent is likely to mean that the bacterial redox protein(s) and P450scc form complexes in lipoprotein structures.

The endogenous reduction of P450scc in the high-speed supernatant proceeds at $k_1 = 0.025\ sec^{-1}$, i.e. 20-25 times slower than in a reconstituted system including Adx and AdR at optimal stoichiometry [34, 35]. This may reflect either poor compatibility of P450scc with the bacterial redox protein(s) or shortage of the latter. A plausible redox partner for P450scc in the *E. coli* cells is the bacterial ferredoxin [36], in view of its structural similarity with adrenocortical Adx [37]. The level of ferredoxin expression in wild-type *E. coli* cells is ~0.1% of total cell protein [36]. In molar terms, this is just below the level of P450scc expression attained in this work. Obviously, a higher cytochrome P450scc expression might mask the effects of bacterial redox proteins because of decreasing of the ratio of these proteins to P450scc. Therefore, in such a case the reduction of this enzyme could hardly be observed. Ferredoxin is likely to be reduced by an NADPH-dependent flavoprotein reductase that is related to adrenocortical AdR [38]. In contrast to recombinant supernatant P450scc, P450scc in the *E. coli* membrane fraction could not be reduced in the absence of Adx and AdR. One should therefore think that in the *E. coli* cells the recombinant P450scc is "serviced" by bacterial partners located in the cytosol.

For the membrane and supernatant fractions, we registered very similar levels of P450scc reduction by NADPH through added Adx and AdR (table), which demonstrates the accessibility of the Adx-binding center of P450scc to the natural partner in both fractions.

P450scc in the high-speed supernatant combined with bacterial redox proteins are able to convert 22R-hydroxycholesterol into pregnenolone (Fig. 5, curves *2* and *3*). Curve *3* shows the inhibitory effect of Emulgen on NADPH-dependent transformation of cholesterol by the supernatant alone. The figure shows HPLC profiles demonstrating accumulation of pregnenolone as the only

reaction product (converted to progesterone for experimental convenience). These data further confirm the functional coupling of P450scc with bacterial redox protein(s).

In the absence of Adx and AdR, the turnover number for conversion of 22R-hydroxycholesterol into pregnenolone was 0.5 and 2.6 min^{-1} for preparations 3 and 4 (table). Upon addition of Adx and AdR, the P450scc activity in the supernatant (preparation 4) reached 27 min^{-1}, which corresponded to the activity of authentic P450scc in the presence of Adx and AdR. Using [^{3}H]cholesterol as a substrate, we also detected formation of pregnenolone. These data once again suggest that cytochrome P450scc can cooperate with bacterial redox proteins in the reaction of cholesterol transformation. In this case the turnover number for preparation 4 was 0.35 min^{-1}, seven times lower than that of the system reconstituted from isolated P450scc, Adx, and AdR at optimal stoichiometry.

Thus, we have found no evidence for the existence of a genuine soluble cytochrome P450scc in *E. coli* cells producing this foreign protein. Our data suggest that the insertion of nascent P450scc polypeptide into the bacterial plasma membrane is quite non-uniform, which is most probably caused by membrane heterogeneity [39, 40]. As a result, disruption of the membrane produces fragments of different composition and properties. Such are the >400-kD lipoprotein particles containing native P450scc in the conventional high-speed supernatant.

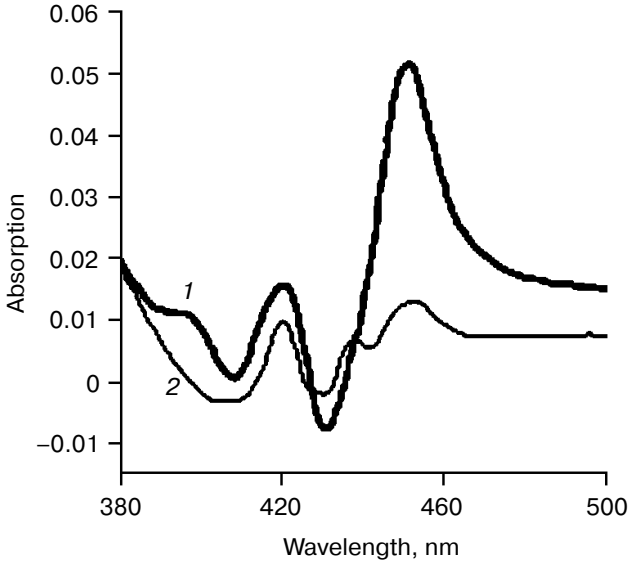

Fig. 4. The CO difference spectra of the *E. coli* high-speed supernatant (120,000*g*, 1 h) after addition of NADPH in the absence (*1*) and in the presence (*2*) of Emulgen 913 (0.3%). The spectra were registered 15 min after addition of NADPH in the absence of Adx and AdR.

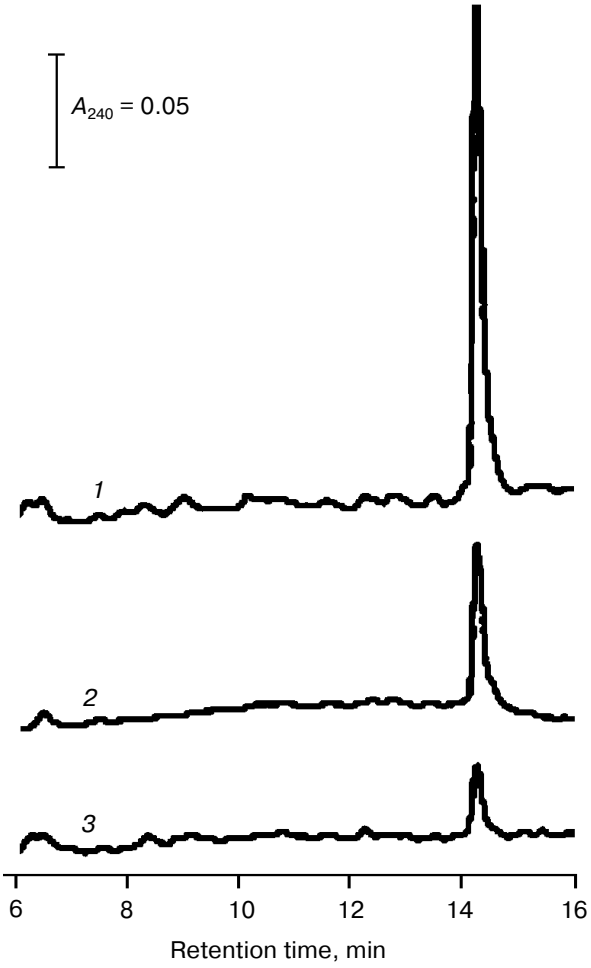

Fig. 5. Cholesterol side-chain cleavage activity of the *E. coli* high-speed supernatant. The sample was incubated with cholesterol in the presence of NADPH, Adx, and AdR (*1*), NADPH only (*2*), or NADPH and Emulgen 913 (*3*). The reaction was continued for 10 min (*1*) or 30 min (*2*, *3*).

Inasmuch as functional cytochrome P450scc is detected in *E. coli* cells only as a constituent part of plasma membrane fragments, one may conclude that the maturation of this protein is obligatorily coupled with its insertion into the bacterial plasma membrane. This is in line with earlier data from our laboratory [41, 42] according to which directed translocation of CYP11A1 into the mitochondrial matrix makes it incapable of proper folding into a catalytically active form.

A comparative study of P450scc functions in the high-speed supernatant surprisingly revealed that the foreign protein could be reduced in the absence of added Adx and AdR. Besides, this fraction proved capable of converting cholesterol or 22R-hydroxycholesterol into pregnenolone. These data suggest that in the *E. coli* cells the recombinant cytochrome P450scc can cooperate with bacterial partners in the cytosol. The cholesterol side-chain cleavage activity in the high-speed supernatant is

~10% of that in the P450scc/Adx/AdR system reconstituted at optimal stoichiometry. As far as we know, the above results illustrate for the first time the possibility of coupling the mammalian cytochrome P450scc with the *E. coli* electron transfer chain.

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