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Coexpression of All Constituents of the Cholesterol Hydroxylase/Lyase System in *Escherichia coli* Cells

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Abstract—Using the pTrc99A/P450scc vector, a plasmid was constructed in which cDNAs for cytochrome P450scc, adrenodoxin reductase, and adrenodoxin are situated in a single expression cassette. This plasmid was shown to direct the synthesis of all the above proteins in *Escherichia coli*. Their localization in the *E. coli* cells and stoichiometry were determined. Cell homogenates exhibited cholesterol hydroxylase/lyase activity, due to catalytically active forms of all three proteins. Thus, the full set of constituents of the mammalian cholesterol hydroxylase/lyase system was shown to be synthesized in bacterial cells for the first time.

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Conversion of cholesterol into pregnenolone is a first and rate-limiting step in the biosynthesis of steroid hormones. This reaction is mediated in mitochondria of adrenal cortex by cytochrome P450scc, which receives electrons from NADPH via two intermediates—adrenodoxin reductase (AdR) and the iron-sulfur protein adrenodoxin (Ad) [1].

Cytochrome P450scc, an integral protein of the inner mitochondrial membrane, is oriented in the membrane in such a way that its catalytic center faces the matrix. It is thus accessible to Ad, a soluble matrix protein [1]. Ad, in turn, interacts with AdR, which is detected in both the matrix and the inner mitochondrial membrane [2].

The stoichiometry of the components of the cholesterol hydroxylase/lyase (CH/L) system is still not determined with certainty. Usanov and coworkers have shown that cytochrome P450scc, AdR, and Ad are present in mitochondria of bovine adrenal cortex in ratio 2.9 : 1 : 7.7 [3], while Hanukoglu et al. reported ratio 8 : 1 : 3 [4].

Notwithstanding the vigorous exploration of the CH/L system in recent years, many important problems concerning its formation and functioning remain unresolved. Those deal, in particular, with stoichiometry and potential ability to form a functional triple complex P450scc·Ad·AdR [5, 6]. The use of transgenic yeast and bacteria with regulated expression of the components of the CH/L system may help answer these questions.

At present, there is only published work concerned with coexpression of mature P450scc, AdR, and Ad in the yeast *Saccharomyces cerevisiae* [7]. Such transgenic yeast possesses an ability to convert ergosta-5-eneol into pregnenolone and progesterone. This study, however, elucidates neither the problem of stoichiometry adjustment of the multienzyme system, nor the mechanism of its functioning.

A radically new approach addressing heterologous expression of CH/L system is the creation of fusion proteins built of distinct and interacting catalytic domains of P450scc, AdR, and Ad. In that way, Harikrishna et al. detected catalytic activity (but not specific activity) of the synthesized in COS-1 cells fusion protein composed of P450scc, AdR, and Ad [8]. Later on [9, 10], expressed in *E. coli* CH/L fusion, with catalytic domains being connected by short (2-5 amino acid residues) linkers, was shown to possess low activity, as compared to the system built of the separate constituents. At least in part this

Abbreviations: Ad) adrenodoxin; AdR) adrenodoxin reductase; CH/L) cholesterol hydroxylase/lyase; DTT) 1,4-dithiothreitol; IPTG) isopropyl-β-D-thiogalactoside; P450scc) cytochrome P450scc; PMSF) phenylmethylsulfonyl fluoride; RBS) ribosome-binding site.

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result was explained by steric hindrance for interaction of active centers of particular domains.

A goal of the present work was to construct a vector allowing simultaneous expression of cDNA for P450scc, AdR, and Ad in *E. coli*. This will deliver a useful tool to study catalytic properties of cytochrome P450scc and its functional peculiarity in heterologous systems, and it may help to obtain transgenic bacterial cells efficiently converting cholesterol and/or its analogs into pregnenolone.

MATERIALS AND METHODS

Reagents used in the work are: 22(R)-hydroxycholesterol, δ-aminolevulinic acid, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, cholesterol oxidase, $NADP^+$, isopropyl-β-D-thiogalactoside (IPTG), *o*-dianisidine, anti-rabbit antibody conjugated with horseradish peroxidase (Sigma, USA); restriction endonucleases, Klenow fragment, T4 DNA polymerase, T4 DNA ligase, and kit for DNA elution (Fermentas, Lithuania). LB- and TB-media for propagation of bacteria were prepared using reagents from Difco (USA). Nitrocellulose membranes for protein transfer were Hybond-C extra from Amersham (USA).

Primary antibodies against bovine cytochrome P450scc, AdR, and Ad as well as P450scc, AdR, and Ad isolated from bovine adrenal cortex [6, 11] were kindly provided by V. M. Shkumatov (Institute of Physico-Chemical Problems, Belorussian State University, Belarus). A kit for measurement of progesterone content by the method of enzyme-linked immunosorbent assay (ELISA) was a generous gift of A. G. Prjadko (Institute of Bioorganic Chemistry of the Belorussian Academy of Sciences, Belarus).

Bacterial strain and plasmids. *Escherichia coli* strain JM-109 (Promega, USA) and vector pTrc99A containing hybrid *trp/lac(trc)* promoter [12] were used in this work. Plasmid pTrc99A/P450scc containing cDNA for mature bovine cytochrome P450scc [13] was kindly provided by M. R. Waterman (University of Texas Southwestern Medical Center, Dallas, USA).

Plasmid pTrc99A/P450scc/AdR containing cDNAs for bovine cytochrome P450scc and human adrenodoxin reductase as a single expression cassette was used as a starting vector to construct a plasmid for coexpression of the three CH/L system proteins. In order to obtain the latter plasmid, two initial plasmids pTrc99A/P450scc and pTrc99A/AdR with cDNA for separate proteins made previously in our laboratory were used.

The DNA insert encoding AdR with ribosome-binding site (RBS) in front was excised from pTrc99A/AdR plasmid with restrictases *Sse8387*I and *Mbi*I. This fragment was cloned into pTrc99A/P450scc plasmid, from which a small fragment was excised in advance with *Sse8387*I and *Sma*I, so that RBS and AdR cDNA were inserted into the expression cassette beyond cDNA for cytochrome P450scc.

To express Ad cDNA alongside the cDNAs for AdR and cytochrome P450scc as a single transcription unit, pTrc99A/P450scc/AdR vector was treated with *Sse8387*I endonuclease and T4 DNA polymerase. The insert coding for mature human adrenodoxin with RBS in front of it was excised with restrictases *Pst*I and *Mbi*I from pTrc99A/Ad plasmid, previously constructed in our laboratory, treated with T4 DNA polymerase, and inserted into the vector.

Escherichia coli cells were transformed by electroporation. The recombinant plasmid obtained (Fig. 1) was quite stable—the ability of the strain bearing this plasmid to synthesize the mentioned proteins was preserved for nine months.

To express the recombinant proteins, the cells of individual colonies were grown overnight in LB medium containing ampicillin (250 μg/ml) diluted 1 : 200 with TB medium and growth was continued at 37°C for 3-4 h. Synthesis of recombinant proteins was then induced by an addition of IPTG to 1 mM, and the cell growth was continued in the presence of ampicillin (100 μg/ml) and δ-aminolevulinic acid (0.5 mM) for 48 h at 22-24°C with constant shaking (140 rpm).

Fractionation of *E. coli* cells. The cells were collected by centrifugation (7500*g*, 10 min, Beckman J2-21, USA) and washed with buffer containing 10 mM HEPES (pH 7.2), 150 mM NaCl, and 5 mM $MgSO_4$. Pellet after a second centrifugation (7500*g*, 10 min) was suspended in

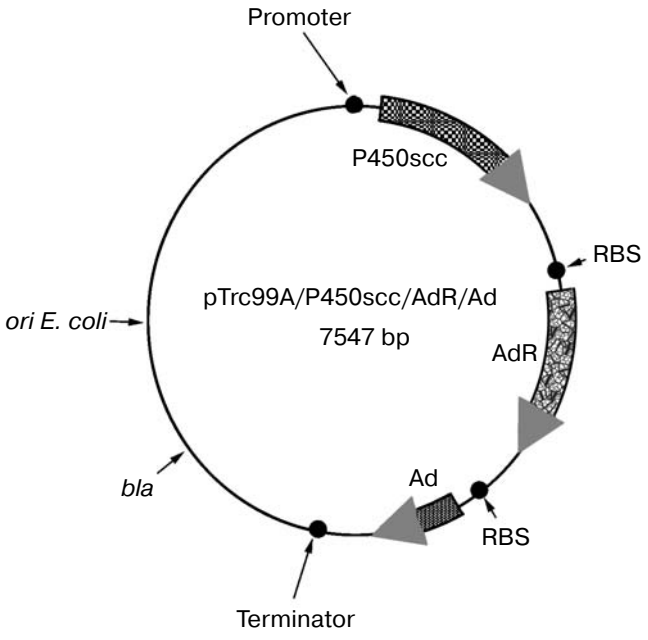

Fig. 1. Diagram of pTrc99A/P450scc/AdR/Ad plasmid containing cDNA for cytochrome P450scc, AdR, and Ad in a single expression cassette.

20 mM sodium phosphate buffer (pH 7.4) containing 5 mM $MgSO_4$, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 4 mM 1,4-dithiothreitol (DTT). The cells were disrupted in a French press (maximum pressure 16,000 psi).

Unbroken cells and debris were removed by centrifugation (31,000*g*, 40 min), and the supernatant was subjected to ultracentrifugation (150,000*g*, 2.5 h, SW 50 rotor, Beckman L2-65B, USA). The pellet was suspended in 50 mM sodium phosphate buffer (pH 7.0). Supernatant and pellet were considered to be cytoplasmic fraction and inverted membrane vesicles, respectively.

Both fractions were subjected to SDS-PAGE in 10 or 15% gel and immunoblotting using antisera (IgG fraction) to P450scc, AdR, or Ad. Each well was loaded with 60 μg of total protein of a fraction. Protein in fractions was measured by the Lowry method.

Assay of CH/L activity. Cell homogenates (0.25-1 mg of total protein) were preincubated for 20 min with 22(R)-hydroxycholesterol (25 nmol) in 30 mM sodium phosphate buffer (pH 7.2) containing 0.05% Tween-20 at 25°C in 0.5 ml volume. When necessary, reaction mixture was also supplemented with 0.5 nmol of isolated cytochrome P450scc, AdR, or Ad (or their mixture). The reaction was initiated by an addition of NADPH-regenerating system (final concentrations: 2 mM $NADP^+$, 10 mM glucose-6-phosphate, and 1 U/ml glucose-6-phosphate dehydrogenase). Resulting pregnenolone was oxidized to progesterone with cholesterol oxidase, and steroids were extracted with ethyl acetate. The precipitate obtained after evaporation of extracts in a vacuum evaporator (Speed Vac Concentrator, Savant, USA) was dissolved in a buffer following the protocol described in [14]. Progesterone content was determined using a kit for enzyme-linked immunosorbent assay ELISA-PROGESTERONE.

Miscellaneous. The differential CO-spectrum was monitored in a homogenate of the *E. coli* cells expressing the components of the CH/L system. Using an Aminco DW-2000 spectrophotometer, the spectrum of reduced cytochrome P450scc was recorded after addition of sodium dithionite. Next, CO was passed through the cuvette for 2 min, and the differential spectrum of the reduced cytochrome P450scc with CO was recorded versus the reduced cytochrome P450scc spectrum.

The amount of recombinant proteins in the cell homogenates was determined after SDS-PAGE and immunoblotting following calibration on nitrocellulose membrane. To this end, homogenate proteins and protein standards of predetermined amount were run on SDS-PAGE in parallel. For calibration, isolated P450scc, AdR, and Ad were used in the range 40-200 pmol. Upon SDS-PAGE and protein transfer from gel onto nitrocellulose membrane, the latter was consecutively treated by a primary antibody and a secondary antibody conjugated with horseradish peroxidase. Protein bands were visualized using *o*-dianisidine. The intensity of the bands for homogenate proteins and markers, scanned after staining, was compared using the ScnImage program.

RESULTS AND DISCUSSION

Coexpression and localization of cytochrome P450scc, adrenodoxin reductase, and adrenodoxin in *E. coli* cells. Cytochrome P450scc, AdR, and Ad were expressed in *E. coli* JM109 upon induction of transcription from the pTrc99A/P450scc/AdR/Ad plasmid during 48 h at lowered temperature (22-24°C) that facilitated stepwise accumulation of heterologous proteins with minimal damage to the cells.

A differential CO-spectrum characteristic of native P450scc was registered in homogenates of the cells transformed with pTrc99A/P450scc/AdR/Ad plasmid (Fig. 2).

Cell homogenate and fractions of low-speed pellet (cell debris and inclusion bodies), membranes, and cytosol were analyzed by SDS-PAGE with subsequent immunoblotting. Cytochrome P450scc, AdR, and Ad obtained from bovine adrenal cortex were used as protein standards, and respective fractions of *E. coli* JM109 cells without the pTrc99A/P450scc/AdR/Ad plasmid served as a negative control.

Proteins were shown to be present in homogenates of the cells transformed with pTrc99A/P450scc/AdR/Ad, which bind antibodies raised against cytochrome P450scc, AdR, and Ad. Their molecular masses correspond to those of the protein standards. All three proteins were also found in the low-speed pellet (45-55% of total amount in the cells) likely due to the presence in this fraction of inclusion bodies, formed during expression of heterologous proteins. If an equal amount of total proteins from cytosol and membrane fraction were subjected to SDS-PAGE (see "Materials and Methods"), cytochrome P450scc was revealed only in the latter (Fig. 3a, lane *6*).

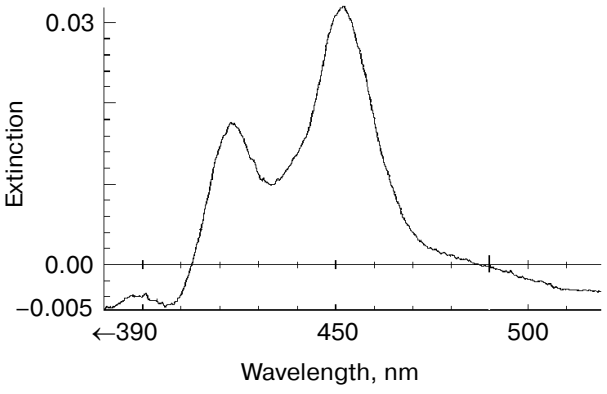

Fig. 2. Differential CO-spectrum of cell homogenate of *E. coli* transformed with pTrc99A/P450scc/AdR/Ad. Sample contained 2.17 mg of homogenate protein in 0.7 ml of 50 mM potassium phosphate buffer (pH 7.4).

This agrees with the data obtained earlier by other researchers [13]. When the protein content in a sample for electrophoresis was increased, P450scc was also detected in cytosol (data not shown).

As to Ad and AdR, analysis of cytosol and membrane fraction showed their presence only in the former fraction (Fig. 3, b and c, lanes *5*). In general, localization of cytochrome P450scc, AdR, and Ad in *E. coli* cells matches their distribution in mitochondria of adrenal cortex (see introductory part).

Content of the protein constituents of the CH/L system in homogenates was determined using calibrated immunoblotting and the ScnImage program. The results are shown in Table 1. They are an average of five experiments concerned with protein expression in *E. coli* and indicate 2.7 : 1 : 6.3 stoichiometry for P450scc, AdR, and Ad, respectively.

When using a tandem plasmid for coexpression of proteins, their content in the cells depends upon several factors such as distance between promoter and coding sequence, size, structure, and stability of mRNA, etc. High content of Ad is probably due to the small size of this protein (12 kD), consistent with the small size of its mRNA. Cytochrome P450scc and AdR are of similar size (56.4 and 51.1 kD, respectively). As cDNA coding for P450scc is closer to the promoter than cDNA for AdR, this may cause a difference in expression level of cytochrome P450scc and AdR. It is known that expression level in *E. coli* of cytochromes P450 27B1, 1A1, 1A2 and NADPH:cytochrome P450 oxidoreductase varies when cDNA for these proteins are placed in different order on polycistronic transcription constructs [15-17]. There may be also other causes of a difference in P450scc, AdR, and Ad content in *E. coli* cells.

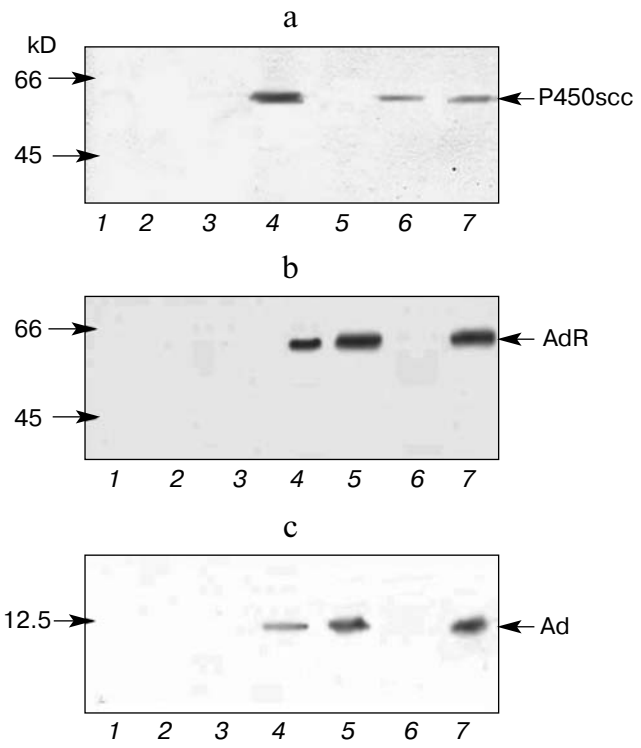

Fig. 3. Expression of cytochrome P450scc (a), adrenodoxin reductase (b), and adrenodoxin (c) in *E. coli* cells transformed with pTrc99A/P450scc/AdR/Ad. Analysis by SDS-PAGE with subsequent immunoblotting. *1-3*) Control *E. coli* JM-109 cells; *4-6*) cells transformed with pTrc99A/P450scc/AdR/Ad plasmid. Cell fractions: low-speed pellet (*1*, *4*), cytosol (*2*, *5*), membrane fraction (*3*, *6*); *7*) proteins isolated from cells of bovine adrenal cortex: cytochrome P450scc (a), adrenodoxin reductase (b), and adrenodoxin (c). Positions of molecular mass markers are shown on the left: bovine serum albumin (66 kD), egg albumin (45 kD), and cytochrome *c* (12.5 kD).

Table 1. Expression of CH/L system proteins in *E. coli* cells transformed with the pTrc99A/P450scc/AdR/Ad plasmid

Component of the CH/L system	Expression level, μmoles per liter of culture
Cytochrome P450scc	0.32
AdR	0.12
Ad	0.76

Interestingly, the ratio of the CH/L system components in *E. coli* transformed with pTrc99A/P450scc/AdR/Ad plasmid fits the data on their stoichiometry in mitochondria of adrenal cortex reported by Usanov et al. [3]. This is surprising, given that the topogenesis of cytochrome P450scc, AdR, and Ad in a natural system includes import of precursors from cytoplasm into mitochondria as well as their proteolytic processing, which may contribute to the final stoichiometry of these proteins. When comparing the stoichiometry of the CH/L system components in natural systems to that observed in the recombinant bacterial cells, it should be kept in mind that, in the latter ones, cytochrome P450scc, AdR, and Ad are partially present in inclusion bodies, i.e., in an inactive form.

Measurement of catalytic activity of the system. In order to assess the activity of the CH/L, we used cell homogenates obtained from *E. coli* JM-109 cells transformed with pTrc99A/P450scc/AdR/Ad and pTrc99A/P450scc plasmids grown for 48 h upon induction of expression. The results of measurement averaged over at least three assays are shown in Table 2. They take into account value scattering, with an activity in the control *E. coli* JM-109 cells being subtracted (which is equal to 0.03 ± 0.02 pmol of pregnenolone per min per mg of homogenate protein).

Activity in homogenates obtained from the cells bearing pTrc99A/P450scc/AdR/Ad did not increase

Table 2. CH/L activity measured in homogenates of recombinant *E. coli* cells

Plasmid used for transformation	Added component	Activity, pmol of pregnenolone per min per mg of protein
pTrc99A/P450scc/AdR/Ad	—	1.6 ± 0.7
	P450scc	1.8 ± 0.9
	AdR	3 ± 0.9
	Ad	1.5 ± 0.5
	AdR + Ad	10.3 ± 0.7
pTrc99A/P450scc	AdR + Ad	14.1 ± 0.8

upon addition of any of the isolated CH/L system proteins (P450scc, AdR, or Ad). Hence, catalytically active forms of all three proteins are created in *E. coli* cells. This is consistent with the data reported for those cases in which cytochrome P450scc [13], AdR [18, 19], and Ad [20] were expressed in *E. coli* separately. Upon addition to cell homogenates of two proteins, AdR and Ad, activity increases up to 10.3 pmol of pregnenolone per min per mg of protein. This value is very close to that obtained under similar condition in homogenates of cells expressing only cytochrome P450scc (14.1 pmol of pregnenolone per min per mg of protein). A substantial increase in the CH/L activity in homogenates of pTrc99A/P450scc/AdR/Ad-bearing cells to which both the AdR and Ad were added indicates that cytochrome P450scc is not a limiting component of the system in the recombinant cells.

Thus, a stable transgenic *E. coli* strain capable of mediating the initial step of biogenesis of steroids in mammals, i.e., conversion of cholesterol into pregnenolone, was first produced in the present work. In future, this strain can be used in fundamental and applied investigations of the CH/L system.

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