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# Comparative Study of Topogenesis of Cytochrome P450scc (CYP11A1) and Its Hybrids with Adrenodoxin Expressed in *Escherichia coli* Cells

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**Abstract**—Hybrid proteins consisting of the mature form of cytochrome P450scc (mP) and adrenodoxin (Ad), attached to either the $NH_2$- or COOH-terminus (Ad-mP and mP-Ad, respectively), were expressed in *E. coli*. Spectral and catalytic properties of P450scc were studied using the membrane fraction of *E. coli* cells. It has been shown that the Ad amino acid sequence attached to the termini of the P450scc-domain neither affects the insertion of a hybrid protein into the cytoplasmic membrane nor influences its heme binding ability. The results suggest that Ad attached to the $NH_2$-terminus does not markedly affect the folding of the P450scc-domain, but cholesterol hydroxylase/lyase activity of the Ad-mP hybrid was found to be much lower than that of the native P450scc enzyme. The modification of the COOH-terminus does not alter the specific P450scc activity, but results in a dramatic increase in the amount of hybrid protein with incorrectly folded P450scc domain.

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*Key words*: cytochrome P450scc, *Escherichia coli*, folding, hybrid proteins

Cytochrome P450scc is a representative of the cytochrome P450 superfamily, performing extremely important functions in living organisms by enabling oxygen-dependent hydroxylation of various substances of exogenous and endogenous origin (drugs, carcinogens, bioregulators, and others). In mitochondria of mammalian adrenal cortex cells, cytochrome P450scc catalyzes the transformation of cholesterol to pregnenolone, a key step in steroid hormone synthesis. The protein is localized in the inner mitochondrial membrane [1], whereas in the study by Shkumatov et al. [2] it was demonstrated that the protein insertion into the membrane is necessary for formation of functionally active enzyme.

*Abbreviations*: Ad) adrenodoxin; CH/L) cholesterol hydroxylase/lyase; DTT) 1,4-dithiothreitol; IMV) inverted membrane vesicles; IPTG) isopropyl-β-D-thiogalactoside; P450scc) cytochrome P450scc; mP) mature form of cytochrome P450scc; PMSF) phenylmethylsulfonyl fluoride.

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At present, there are no data on obtaining a water-soluble form of cytochrome P450scc, and therefore there is no possibility to obtain protein crystals for X-ray analysis. Using computer programs, four cytochrome P450scc models based on the crystal structure of cytochromes P450cam, P450 BM3, P450 2C5, and P450 2B4 have been obtained [3-6]. Despite the low degree of homology between cytochrome P450scc and these proteins, the spatial models of cytochrome P450scc are largely similar to each other, reflecting high conservatism of three-dimensional structure of the cytochrome P450 superfamily. Among the proteins listed above only P450 2C5 is a membrane protein and contains structural elements for interaction with microsomal membrane; therefore, a P450scc model constructed based on P450 2C5 structure is probably the most similar to the actual structure of this protein. It was assumed that mitochondrial and microsomal cytochromes P450 have a similar way of association with membrane through a large hydrophobic domain anchored into a lipid bilayer formed by several non-neighboring regions of the polypeptide chain, among

which F–G loop and A′-helix regions are listed [5, 7]. It has been demonstrated that mutation in the F–G loop region of cytochromes P450 7A1, 27 A1, and 11A1 (P450scc) lead to a change in their intracellular distribution. On this basis, it was assumed that the F–G loop is a membrane-anchoring site for these enzymes [8-10].

Besides the structure of already formed enzyme, it is of interest to study the processes of protein folding into a correct conformation, as well as protein sequence regions and factors determining the efficiency of this process. *In vivo* formation of such complex proteins as cytochrome P450scc includes incorporation of heme, membrane anchoring, and possible interaction with chaperons facilitating protein folding. At present, there are data available on an important role of terminal amino acid sequences in cytochrome P450 folding. Both for mammalian (microsomal and mitochondrial) and bacterial cytochromes P450, it was shown that a conservative N-terminal sequence rich in proline residues (PR sequence, from *proline rich*) plays an important role in the formation of the protein structure [11, 12]. The PR sequence in the cytochrome P450scc molecule, bearing four proline residues at positions 6, 8, 13, and 15, is revealed at the N-terminus after cleavage of the addressing sequence. It was shown that the proline residue at position 13 plays a determining role in cytochrome P450scc folding into native conformation. The replacement of this amino acid residue with alanine results in the formation of a recombinant protein without a characteristic CO difference spectrum [12]. However, data on the role of PR sequence are ambiguous. On one hand, Chen et al. [13] and Kemper [14] suggest a hypothesis that the PR sequence is not only necessary for a correct protein folding, but also stabilizes the final conformation, interacting with certain regions of the protein molecule. On the other hand, Kusano et al. [11] demonstrated that the PR sequence plays an important role only in the protein folding process, but is not necessary for maintaining the structure of already folded protein. Thus, for instance, the cleavage of the PR sequence after completing the formation of microsomal cytochrome P450c17 does not lead to a change in spectral features of the protein. Regarding the role of the C-terminal sequence in topogenesis of cytochromes P450, not much information is available at present. It was found that the COOH-terminus of human cytochrome P450 46A1 also contains a proline-rich region (five proline residues). However, during the study of a recombinant P450 46A1 variant containing a deletion at the COOH-terminus, it turned out that this region (contrary to N-terminal PR sequence) has no effect on polypeptide chain folding, but facilitates the formation of protein dimers [15]. Considering the diversity of the cytochrome P450 superfamily, one can assume that despite the similarity of final structures, the factors determining the formation of molecules, as well as conditions necessary for formation of various cytochromes, can differ dramatically.

Therefore, despite active study of cytochromes P450, the investigation of topogenesis of these proteins is still important, in particular, elucidation of molecule regions playing a determining role in the formation and maintenance of catalytically active protein. All data available at present on the role of N- and C-terminal sequences in cytochrome P450 folding (and, in particular, P450scc) were obtained during the study of recombinant proteins with deleted or mutated terminal regions [11-15]. The study of hybrid proteins, in which the elements necessary for correct folding are shielded or deformed as a result of fusion of the studied protein with another protein, can provide a new source of data about the formation of protein structure and the role of the N- and C-terminal sequences of the molecule in this process.

In the present work, we studied the properties of recombinant cytochrome P450scc whose terminal regions were loaded with the relatively small soluble protein adrenodoxin. The fusion proteins were synthesized in *E. coli* cells, for which it was demonstrated that expression of the gene of mature cytochrome P450scc form results in formation of catalytically active protein [16]. By analysis of changes in P450scc properties in hybrid proteins, we showed that the C-terminal region is more important for ensuring the correct folding and protein stability than the N-terminal region. However, contrary to the C-terminal region of the P450scc molecule, the N-terminus is apparently involved in maintaining the structure of the catalytically active enzyme.

## MATERIALS AND METHODS

**Strains, plasmids, and materials used.** This work was carried out using JM-109 *E. coli* cells (Promega, USA) and pTrc99A plasmid with a hybrid promoter *trp/lac/trc* [17]. The pTrc99A/mCYP11A1 plasmid, containing nucleotide sequence encoding for a mature form of cytochrome P450scc (mP) from bovine adrenal cortex, was kindly provided by Dr. Waterman (Vanderbilt University, USA) [16]. Plasmids for expression of hybrid proteins mP-Ad and Ad-mP were previously obtained in our laboratory [18]. Growth media were prepared using reagents supplied by Difco (USA). The immunoassay kit for determination of progesterone concentration, IFA-PROGESTERON, was provided by A. G. Pryadko (Institute of Bioorganic Chemistry, Belorussian Academy of Sciences). Recombinant proteins were identified using primary antibodies against bovine cytochrome P450scc and Ad (kindly provided by V. M. Shkumatov, Institute of Physico-Chemical Problems, BGU, Belorussia) and conjugate of rabbit antibodies with horseradish peroxidase (Sigma, USA).

**Expression of mP genes and hybrid Ad-mP and mP-Ad variants in *E. coli* cells.** Expression of mP genes and hybrid Ad-mP and mP-Ad variants in *E. coli* cells was performed as described previously [16, 18]. Competent

bacterial cells were transformed with recombinant plasmids using a standard procedure [19]. Gene expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) (0.5 mM) to the growth medium. As an additional source of heme, δ-aminolevulinic acid was used (0.5 mM). The culture was incubated for 40 min at 24°C under constant stirring (140 rpm).

**Fractionation of *E. coli* cells.** Bacterial cells from 1 liter of culture with $A_{600} \approx 6$-7 were pelleted by centrifugation (7500*g*, 10 min) and washed with buffer (10 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM $MgSO_4$). Then the cells were resuspended in a buffer containing 20 mM sodium phosphate, pH 7.4, 5 mM $MgSO_4$, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 4 mM 1,4-dithiothreitol (DTT) and disintegrated in a French press (maximal pressure 16,000 psi). The homogenate was centrifuged for 15 min at 14,000*g* for removal of large cell fragments and inclusion bodies formed during the synthesis of heterologous protein. Cleared supernatant was subjected to high speed centrifugation at 140,000*g* for 2.5 h. The precipitate constituting inverted membrane vesicles (IMV) was resuspended in 50 mM sodium phosphate buffer, pH 7.4, mixed with glycerol (final concentration 20%), and stored at –20°C.

For carbonate extraction, IMV (50 μg of protein) were resuspended in 100 μl of freshly prepared buffer (10 mM Hepes, pH 7.2, 0.1 M $Na_2CO_3$) and incubated on ice for 30 min. The membrane fraction was precipitated in an Airfuge ultracentrifuge (Beckman, USA) at 150,000*g* for 30 min using an A100 rotor.

**Spectral analysis.** Sodium dithionite was added to the IMV sample containing 1 mg of protein in 700 μl of 50 mM sodium phosphate buffer, pH 7.2, and the spectrum of reduced P450scc was recorded. After that CO was bubbled through the cuvette for 2 min, and the spectrum of reduced complex of P450scc with CO was recorded against the spectrum of reduced P450scc. Spectrum of the control sample, an analogous IMV preparation (1 mg of protein) from *E. coli* cells without recombinant DNA, was subtracted from the above spectrum. The content of native and denatured protein forms was calculated from the obtained CO difference spectrum using the following molar extinction coefficients: $\varepsilon_{450\text{-}490} = 91\ mM^{-1}\cdot cm^{-1}$ and $\varepsilon_{420\text{-}490} = 111\ mM^{-1}\cdot cm^{-1}$, respectively [20].

**Measuring activity of cholesterol hydroxylase/lyase system.** To the IMV fraction (0.5 mg of total protein) 22R-hydroxycholesterol (25 nmol), adrenodoxin (0.5 nmol), and adrenodoxin reductase (0.5 nmol) were added and incubated for 20 min in 30 mM sodium phosphate buffer (pH 7.2) with 0.05% Tween-20 at 25°C in a total volume of 0.5 ml. The reaction was induced by addition of NADPH-generating system (final concentrations: 2 mM $NADP^+$, 10 mM glucose-6-phosphate, and 1 unit/ml glucose-6-phosphate dehydrogenase), allowed to proceed for 30 min at 37°C, and then stopped by heating in a boiling water bath for 2 min. Formed pregnenolone was oxidized to progesterone by adding cholesterol oxidase (0.5 unit in 100 mM sodium phosphate buffer, pH 7.2, with 0.05% Tween-20) to the sample and incubating for 1 h at 37°C. The steroids were extracted with ethyl acetate three times, and the precipitate obtained after evaporation of the extracts on a vacuum rotor (SpeedVac Concentrator; Savant, USA) was dissolved in the buffer for determination of progesterone concentration. The progesterone concentration was measured using an IFA-PROGESTERON immunoassay kit.

**Other methods.** Protein concentration was measured by the Lowry method [21]. Standard procedures were used for identification of cytochrome P450scc in cell fractions by SDS-PAGE in 10% gel [22] and Western blotting [23] using antiserum (IgG fraction) against bovine P450scc or adrenodoxin.

## RESULTS

**Expression of genes of hybrid Ad-mP and mP-Ad proteins in *E. coli* cells.** *Escherichia coli* cells were transformed with plasmids bearing the genes of recombinant mP, mP-Ad, and Ad-mP proteins, expression was induced, and the cell culture grown for 48 h at 24°C. As follows from the literature, this incubation temperature is optimal for accumulation of recombinant P450scc [16]. Electrophoretic analysis of cell homogenates in polyacrylamide gel followed by immunoblotting with antibodies against P450scc or adrenodoxin revealed that transformed cells express the genes of the recombinant pro-

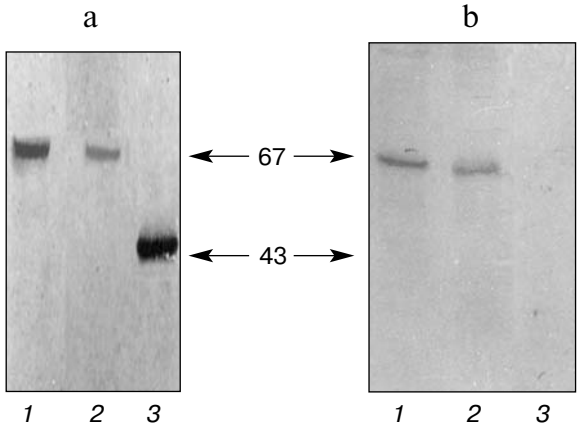


**Fig. 1.** Expression of mP and Ad-mP and mP-Ad hybrid variants in *E. coli* cells. SDS-PAGE followed by immunoblotting of cell homogenates 48 h after addition of IPTG. a) Staining with antibodies against P450scc; b) staining with antibodies against adrenodoxin. *1*) Homogenate from JM109 cells transformed with pTrc99A/Ad-mP plasmid; *2*) homogenate from JM109 cells transformed with pTrc99A/mP-Ad; *3*) homogenate from JM109 cells transformed with pTrc99A/mP. Arrows show the position of protein molecular weight markers (kD).

Spectral and catalytic properties of cytochrome P450scc and Ad-mP and mP-Ad hybrid proteins in IMV preparation

| Parameter | P450scc variants | | |
|---|---|---|---|
| | mP | Ad-mP | mP-Ad |
| Amount of P450 (nmol/mg IMV protein) | 0.14 ± 0.03 | 0.08 ± 0.03 | 0.02 ± 0.005 |
| Amount of P420 (nmol/mg IMV protein) | 0.04 ± 0.012 | 0.09 ± 0.02 | 0.09 ± 0.03 |
| P450/P420 | 3.5 ± 0.6 | 0.9 ± 0.4 | 0.2 ± 0.06 |
| Total amount of heme protein P450 + P420 (nmol/mg IMV protein) | 0.18 ± 0.031 | 0.17 ± 0.05 | 0.11 ± 0.035 |
| Amount of protein by immunoblotting (nmol/mg IMV protein) | 0.24 ± 0.08 | 0.21 ± 0.08 | 0.16 ± 0.06 |
| Efficiency of heme incorporation (%) | 75 ± 10% | 80 ± 12% | 70 ± 13% |
| Specific activity (nmol progesterone/nmol P450 form × $min^{-1}$) | 3.4 ± 1.4 | 0.98 ± 0.34 | 4.8 ± 1.7 |

Note: The data are expressed as mean ± standard deviation of three independent experiments.

teins (Fig. 1). Protein molecular weights (mP450scc, 53 kD; mP-Ad and Ad-mP, 65 kD) and their immunospecificity were as expected. According to immunoblotting results, the level of Ad-mP expression was comparable with the level of mP450scc expression, whereas the level of mP-Ad expression was slightly lower.

To determine cellular localization of Ad-mP and mP-Ad hybrid proteins, cell homogenates were separated into membrane and soluble fractions. It was shown previously that during the expression of mature P450scc form in *E. coli* cells, the protein is found predominantly in the membrane fraction [2]. Analysis of cellular distribution of synthesized hybrid Ad-mP and mP-Ad proteins revealed that they also are localized in the membrane fraction; therefore, membrane protein preparations were used for a comparative study of the recombinant proteins. Ad-mP protein content in IMV fraction, estimated by immunoblotting results, was comparable with the content of mP450scc, whereas mP-Ad content was slightly lower (see the table).

For determination of whether the recombinant proteins are incorporated into the plasma membrane of *E. coli* cells, IMV fractions were treated with 0.1 M $Na_2CO_3$. As in the case of non-modified variant [24], it was found that mP-Ad and Ad-mP proteins upon treatment with 0.1 M $Na_2CO_3$ are virtually not washed out from the membranes (Fig. 2). This indicates that these proteins are incorporated into the membrane, but not associated on its surface.

Therefore, the presence of additional amino acid sequences of adrenodoxin at the ends of the cytochrome P450scc molecule does not hinder its insertion into the bacterial plasma membrane.

**Effect of modifications of the terminal regions on mP450 folding.** Cytochrome P450scc with bound heme has a characteristic CO difference spectrum. The presence of a peak at 450 nm in the protein CO difference spectrum indicates that cytochrome P450 with associated heme acquires a correct conformation (native form); incorrectly folded protein with bound heme is characterized by a CO difference spectrum with a peak at 420 nm (denatured form). Total amount of heme protein can be calculated from the sum of native and denatured form, using the corresponding extinction coefficients (see "Materials and Methods"). Figure 3 shows the CO difference spectra for membrane fraction preparations containing mP, Ad-mP, and mP-Ad. It should be noted that the mP-Ad spectrum could not have been registered earlier using cell homogenate for analysis [18] due to low specific content of the hybrid protein (0.01 nmol per mg protein). As follows from the table, protein amount determined based on results of immunoblotting for mP, Ad-mP, and mP-Ad was higher than the amount of heme pro-

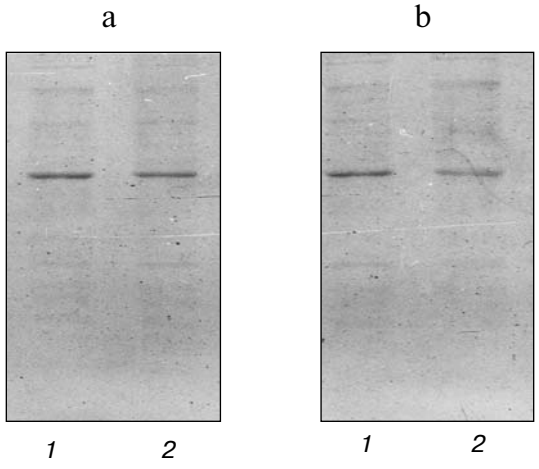


**Fig. 2.** Results of treating IMV containing Ad-mP (a) and mP-Ad (b) hybrid proteins with 0.1 M $Na_2CO_3$. *1*) IMV fraction before the treatment; *2*) pellet after treatment with 0.1 M $Na_2CO_3$. Staining with anti-adrenodoxin antibodies.

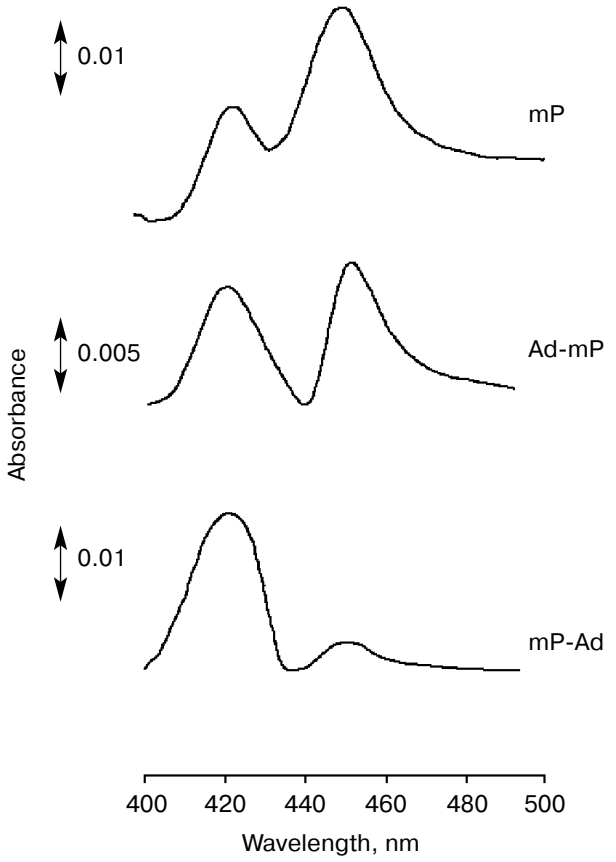


**Fig. 3.** CO difference spectra of IMV fractions obtained from cells expressing mP, Ad-mP, and mP-Ad. The data obtained after subtracting the spectrum of control IMV that did not contain recombinant proteins.

tein calculated from the CO difference spectrum, indicating that not all recombinant protein molecules contain heme. However, the amount of molecules with bound heme is approximately the same for the three protein variants, 70-80%. Therefore, the incorporation of heme in the hybrid molecules occurs with the same efficiency as in the case of non-modified P450scc.

The table also lists the ratio between the native and denatured protein forms, which is an indicator of the efficiency of forming the correctly folded heme protein from the molecules with bound heme. In the case of mP this ratio is, on average, equal to 3.5, and for the hybrid Ad-mP and mP-Ad variants it is 0.9 and 0.2, respectively. Thus, in the case of Ad-mP approximately half of the heme protein molecules are folded correctly, and in the case of mP-Ad only one fifth. Consequently, both hybrid variants take the correct conformation with a significantly lower probability than mP.

**Functional state of cytochrome P450scc within the hybrid molecules.** To elucidate the effect of the presence of additional sequences at the ends of the molecule on functioning of P450scc active site, we measured the cholesterol hydroxylase/lyase (CH/L) activity in IMV preparations containing mP, mP-Ad, and Ad-mP. As seen from the table, the specific enzymatic activity calculated per amount of functional cytochrome P450scc present in the sample, in the case of hybrid Ad-mP protein was only about 30% of CH/L activity of non-modified protein, and in the case of mP-Ad it was comparable with the activity of mP.

These data indicate that the presence of adrenodoxin at the $NH_2$-end of P450scc sequence (Ad-mP) had an effect not only on polypeptide chain folding, but also on functioning of the active site. On the contrary, loading of the C-terminal P450scc sequence in the case of mP-Ad dramatically complicates the folding process; however, if the protein has folded into a correct conformation, such modification has no effect on enzyme catalytic activity.

## DISCUSSION

Earlier we have shown [2] that during the synthesis of cytochrome P450scc lacking the N-terminal addressing presequence the heme-containing protein form is found only in the cell membrane fraction. This indicates that the formation of catalytically active protein form is conjugated with the insertion of its polypeptide chain into the plasma membrane. Consequently, this membrane P450scc form can serve as an adequate model for studying the folding process of newly synthesized CYP11A1 polypeptide chain. In the present work an attempt was made to elucidate the role of N- and C-terminal sequences in polypeptide chain folding, accompanied by the formation of heme-binding site, as well as substrate (cholesterol and its analogs)- and adrenodoxin (the electron donor)-binding sites.

Since adrenodoxin is a distinct hydrophilic protein, bearing an overall negative charge and having a molecular weight of 12 kD [25], it was of interest to determine how its attachment to the N- and C-terminal sequences of the polypeptide chain will influence the spectral and catalytic properties of the hybrid molecules, and also their ability to incorporate into membrane. Considering the properties of Ad mentioned above, one could expect that, acting as a float in regards to the P450scc domain, Ad would have an effect on these parameters of hybrid molecules. It turned out that the presence of the Ad amino acid sequence at the ends of the P450scc domain has no effect on its insertion into the membrane (according to carbonate test data). Therefore, the $NH_2$- and COOH-terminal do not play a significant role during the insertion of the polypeptide chain of native P450scc into the membrane. This corresponds to the assumption [5, 10] according to which a critical role in this process belongs to the F–G loop in the region of amino acid residues 207-230. The character of interaction of the polypeptide chain region with the lipid bilayer is not yet determined. It is only

known that the F–G loop does not contain a region enriched with hydrophobic amino acid residues that could be long enough for a crossover through the entire lipid bilayer.

Dramatic difference was found during the comparative study of spectral properties of mP, Ad-mP, and mP-Ad in IMV preparations. It turned out that with approximately equal content of the heme-containing protein fraction (by the sum of P420 and P450 forms), the above variants differ in the ratio of these forms (see Fig. 3). The comparison of data for Ad-mP and mP-Ad with the data for mP shows that the presence of Ad at the $NH_2$- and COOH-termini of the P450scc domain sequence significantly decreases the probability of formation of native heme-containing site, whereas this effect is more profound in the case of the mP-Ad variant, bearing Ad at the COOH-terminus. It is probable that the additional adrenodoxin domain in the case of Ad-mP decreases the mobility of the N-terminal sequence and restricts the involvement of a proline-rich region in the protein folding process. The presence of Ad at the COOH-end of the sequence dramatically hampers the formation of native (P450 form) of the heme-containing site, which can be associated with the location of the heme-containing site in the COOH-terminal region of the cytochrome P450scc molecule [26]. An alternative can be high lability of the native heme-containing site (P450 form) in the hybrid proteins, in particular mP-Ad. Indeed, a considerable amount of P420 form is found even in native P450scc (mP) in IMV, which can be due to partial protein denaturation in the process of preparing IMV from *E. coli* cell lysate (Fig. 3).

The state of the heme-containing site is not the only characteristic of cytochrome P450scc polypeptide chain folding during the incorporation into the membrane. Additional information about this process can be provided by estimation of catalytic properties of the protein. In the present study only preliminary data are given. Previously we have shown [18] that the Ad-mP variant exhibits low catalytic activity in the system containing only the substrate (22R-hydroxycholesterol) and adrenodoxin reductase (AdR). However, the activity of hybrid protein substantially increases upon addition of the excess of exogenous P450scc, reaching a value similar to the one characteristic for the system reconstructed from individual isolated proteins, cytochrome P450scc, Ad, and AdR. Therefore, in the present work all measurements of cholesterol hydroxylase/lyase activity were carried out in the presence of excess amounts of exogenous Ad and AdR. The comparison of specific activities of mP and mP-Ad calculated for each protein in regard to the content of P450 form yielded unexpected results (see table). It turned out that the Ad-mP variant with relatively high content of P450 form exhibits low activity, accounting for only 30% of the activity of native protein (i.e. mP) within IMV. Low activity of this protein is probably due to either disruption of P450scc interaction with exogenous Ad or deformation of the substrate-binding site during the formation of P450scc as part of a hybrid protein. Regarding the mP-Ad hybrid, estimation of its activity faces significant difficulties because of the extremely low content of P450 form. Nevertheless, the available data imply that the specific activity of mP-Ad variant is comparable with the activity of native mP.

The existence of the heme binding site in Ad-mP and mP-Ad hybrids indicates that folding of the polypeptide chain of mature cytochrome P450scc form is not initiated at the N- or C-terminal sequences, but starts from a nucleus, which can be, for instance, the heme binding region, as in the case of cytochrome *c* [27]. This is in agreement with the theory of the folding nucleus, necessary for subsequent folding [28], with an addition that in the case of P450scc the formation of the nucleus is somehow coupled to the interaction of polypeptide chain with *E. coli* plasma membrane (or inner mitochondrial membrane in mammalian cells), since heme-containing form is not found in the soluble protein fraction upon mCYP11A1 expression in *E. coli*. At the same time, the present work shows that modification of the terminal regions of the P450scc polypeptide chain can have an effect on the formation of the native heme binding site and catalytic activity of the protein.

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