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Role of C-Terminal Sequence of Cytochrome P450scc in Folding and Functional Activity

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Abstract—To elucidate the role of Arg472 and C-terminal sequence of the mature form of cytochrome P450scc, a mitochondrial cytochrome P450, in the present work we have performed sequential removal of the C-terminal amino acid residues of the hemeprotein and evaluated their functional role in folding and catalysis. The removal of 2, 4, 7, or 9 amino acid residues (cytochrome P450scc mutants Δ2, Δ4, Δ7, and Δ9) does not significantly affect the physicochemical properties of the truncated forms of cytochrome P450scc, but results in significant increase in the expression level of the hemeprotein in *Escherichia coli* (Δ4 cytochrome P450scc mutant). However, removal of 10 C-terminal amino acid residues (Δ10 cytochrome P450scc) of mature form of cytochrome P450scc (replacement of codon for Arg472 for stop-codon) is followed by loss of the ability for correct folding in *E. coli*. Based on these data, it is concluded that the C-terminal amino acid residues of cytochrome P450scc (ΔArg472-Ala481) play an important role in correct recombinant protein folding and heme binding by cytochrome P450scc during its expression in *E. coli*, while folding of mitochondrial cytochrome P450scc during its heterologous expression in bacterial cells is more similar to the folding of prokaryotic soluble cytochrome P450's than to microsomal cytochrome P450's.

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Cytochrome P450 represents a super-family of hemeproteins that are widely distributed in all living organisms. Cytochrome P450 catalyzes numerous reactions of biosynthesis and transformation of different physiologically important compounds such as steroids, fatty acids, hormones, xenobiotics including drugs, food additives, and pollutants [1]. Despite the wide diversity and distribution, all eukaryotic cytochrome P450's can be divided to form at least two main classes: microsomal cytochrome P450's (mostly located in the endoplasmic reticulum membranes) and mitochondrial cytochrome P450's (located in the inner mitochondrial membrane) [2].

The most important role cytochrome P450 plays is in the biosynthesis of steroids [3, 4]. Cholesterol side-chain cleavage cytochrome P450—cytochrome P450scc (CYP11A1)—is expressed mostly in steroidogenic tissues, being the terminal oxidase of the cholesterol side-chain cleavage system that participates in transformation of cholesterol to pregnenolone, the main precursor of steroid hormones [5]. During transformation to pregnenolone, cholesterol undergoes three sequential hydroxylation reactions resulting in formation of 22R-hydroxycholesterol, 20,22-dihydroxycholesterol, and finally pregnenolone via the lyase reaction. The conversion of cholesterol to pregnenolone determines the rate of biosynthesis of steroid hormones and is under strict regulatory control [6].

Cytochrome P450-dependent sterol-14α-demethylase (CYP51) is one of the most important enzymes of sterol biosynthesis in pro- and eukaryotes [7]. Cytochrome P450-dependent sterol-14α-demethylase catalyzes the oxidative elimination of the 14α-methyl group of lanosterol and 24-methylene-24,25-dihydrolanosterol in yeasts and fungi, obtusifoliol in plants, and 24,25-dihydrolanosterol in animals with formation of $\Delta^{14,15}$-unsaturated intermediate products of biosynthesis pathways—ergosterol (fungi), phytosterol (plants), and cholesterol (mammals). During the conversion, the substrate undergoes three sequential monooxygenation reactions

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resulting in formation of 14-hydroxymethyl- and 14-formyl-derivatives with subsequent removal of formic acid and formation of a double bond between carbon atoms 14 and 15 [8]. The fact that sterol-14α-demethylase performs the same catalytic function in all biological kingdoms was the basis for the hypothesis that this enzyme was the subject of evolution in the late prokaryotic era (after the appearance of molecular oxygen in the atmosphere) and is the precursor for all eukaryotic cytochrome P450's.

Alignment of the amino acid sequences of sterol-14α-demethylases from different sources such as mammals, plants, fungus, and bacteria indicate that arginine residue Arg448 of cytochrome P450 from *M. tuberculosis* is rather conservative as compared to C-terminal sequences of other representatives of this subfamily of cytochrome P450. The removal of the positive conservative charge in the C-terminal sequence results in inability of water-soluble cytochrome P450 from *M. tuberculosis* (MTCYP51) to express in *E. coli* cells and correctly fold in bacteria. At the same time, the removal of the conservative positive charge in the C-terminal sequence of microsomal eukaryotic homolog (human and *C. albicans* cytochrome P450) does not significantly affect the degree of heterologous expression of these hemeproteins and their folding [9].

As follows from the three-dimensional structure of cytochrome P450 from *M. tuberculosis* [10], the positively charged arginine residue Arg448 is located in close vicinity to the negatively charged carboxylic group of aspartic acid, Asp287 (C-terminal sequence of α-helix J), while the rest of the side chain of this residue is located close to β-sheet 3 (residues 412-415). The alignment of amino acid sequences of mitochondrial cytochrome P450's from the subfamily CYP11 with the C-terminal sequence of CYP51 from *M. tuberculosis* indicates that Arg488 of CYP51 corresponds to Arg472 of cytochrome P450scc, which is rather conservative among the representatives of this subfamily.

The aim of the present work was to elucidate the functional role of the C-terminal sequence of cytochrome P450scc in folding and heme binding during its heterologous expression in *E. coli* by using sequential removal of the C-terminal amino acid residues of the hemeprotein. The results presented in the current paper decisively indicate the functional significance of the C-terminal amino acid residues of cholesterol side-chain cleavage cytochrome P450scc (ΔArg472-Ala481) in correct folding of the recombinant hemeprotein and heme binding by cytochrome P450scc during its expression in *E. coli*.

MATERIALS AND METHODS

Materials. In the present work we used isopropyl-β-D-thiogalactopyranoside (IPTG) (Gibco BRL, USA); yeast extract, peptone, and bactotryptone (Difco, USA); cholesterol, pregnenolone, sodium cholate, Tween 20, Coomassie R-250, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, poly(ethylene glycol) 6000, and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (Serva, Germany); Sepharose 4B, CNBr-activated Sepharose 4B, and DEAE-Sepharose 6B (Pharmacia, Sweden); δ-aminolevulinic acid, phenylmethylsulfonyl fluoride (PMSF), and NADP(H) (Sigma, USA); TSK-gel HW-50 (Toyopearl, Japan); Bio-Gel HTP (Bio-Rad, USA).

Site-directed mutagenesis of cytochrome P450scc. The deletions from the C-terminal sequence were carried out by substitution of the corresponding C-terminal amino acid with stop-codon by using the following reverse primers containing restriction site for restrictases *Pst*I or *Sph*1:

SCC-2, GTTCTGCAGCTAGGGCGGGTCCTGGTTG;

SCC-4, GTTCTGCAGCTAGTCCTGGTTGAAGGGGCGGAAG;

SCC-7, GAACTGCAGCTAAAAGGGGCGGAAGACAAGG;

SCC-9, GAACTGCAGCTAGCGGAAGACAAGGAAG;

SCC-10, TGCATGCAGTCAGAAGACAAGGAAGAT.

To amplify the fragment of cDNA for cytochrome P450scc, we used direct 5′-primer:

GCCTCCTGAAAAGTGAGCAGATGCTCTTGGAGGATG.

Amplification of the fragment of cDNA for cytochrome P450scc by using the above primers was carried out as previously described [11]. After polymerase chain reaction (PCR), reaction products and initial vector containing cDNA for mature bovine cytochrome P450scc were treated with restrictase *Pst*I. Then the original fragment of cDNA for wild type cytochrome P450scc was replaced with amplified fragment containing stop-codon in the corresponding position. The plasmid pTrc99A containing cDNA for mature bovine cytochrome P450scc was generously presented by Prof. M. R. Waterman (Vanderbilt University, USA).

The presence of the desired substitution was monitored by restriction analysis and sequencing on an A377 automatic DNA-sequencer (Applied Biosystems, USA).

Protein expression and purification. The plasmids containing cDNAs for mature bovine adrenodoxin (pBa1159) and adrenodoxin reductase (pBAR 1607) were kindly supplied by Prof. I. Sagara (Kochi Medical

School, Japan). Expression and purification of adrenodoxin and adrenodoxin reductase were carried out as previously described [12].

Expression and purification of truncated forms and full-length cytochrome P450scc from recombinant *E. coli* cells was carried out according to the scheme for preparation of substrate-bound high-spin hemeprotein [13].

Analytical methods. Analysis of protein composition of recombinant cells after expression and monitoring of the purity of purified protein preparations was carried out using SDS-PAGE in 10% gel [14].

Specific staining of gels for peroxidase activity of heme was carried out using *o*-phenylenediamine (OPD) (15 mg in 10 ml of 50 mM sodium phosphate-citrate buffer, pH 5.0) during 30 min with shaking in the dark at room temperature. Then hydrogen peroxide was added to final concentration 30 mM. The staining of gels becomes evident after 3 min and the color is further developed during 20-25 min. The gel was quickly washed in 50 mM sodium phosphate-citrate buffer, pH 5.0, and photographed.

Immunochemical identification of truncated forms of cytochrome P450scc was done by using polyclonal antibodies to bovine cytochrome P450scc [15]. To determine the concentration of cytochrome P450scc, adrenodoxin reductase, and adrenodoxin, the molar extinction coefficients 91 $mM^{-1}\cdot cm^{-1}$ at 393 nm, 11 $mM^{-1}\cdot cm^{-1}$ at 450 nm, and 10 $mM^{-1}\cdot cm^{-1}$ at 414 nm were used, respectively [16, 17].

Spectral characterization of mutant forms of cytochrome P450scc. Spectra were measured using a Shimadzu UV-3000 (Shimadzu, Japan) spectrophotometer. The concentration of active cytochrome P450scc and its denatured form, cytochrome P420, were determined from the reduced (reduced with sodium dithionite) carbon monoxide minus reduced difference spectra (CO-spectra) of the hemeprotein using molar extinction coefficients 91 $mM^{-1}\cdot cm^{-1}$ at 450 nm and 114 $mM^{-1}\cdot cm^{-1}$ at 420 nm [16].

Circular dichroism spectra were measured using a JASCO J-720 spectropolarimeter (JASCO, Japan) under the following experimental conditions: slit, 1 nm; response time, 2 sec; scan speed, 20 nm/min; temperature, 20°C. The data of at least three measurements were accumulated. In the UV region (195-250 nm), the measurements were done using optical pathlength 0.1 cm and concentration of cytochrome P450scc 1 μM in 10 mM potassium phosphate buffer, pH 7.2. During recording of the spectra in the visible region of spectra (300-600 nm) the optical pathlength 0.5 cm and cytochrome P450scc concentration 10 μM were used.

Limited proteolysis of cytochrome P450scc and its mutant forms with trypsin. The limited proteolysis of truncated mutants and wild type full-length cytochrome P450scc was carried out in 50 mM potassium phosphate buffer, pH 7.4, at final concentration of cytochrome P450scc in samples 10 μM at 37°C and molar ratio trypsin/cytochrome P450scc of 1 : 50. The concentration of trypsin was determined spectrophotometrically ($\varepsilon_{280} = 3.5\cdot10^4\ M^{-1}\cdot cm^{-1}$). Trypsinolysis was stopped by adding 4-fold excess (w/w) of soybean trypsin inhibitor. To analyze the products of proteolytic modification of cytochrome P450scc, SDS-PAGE in 12% gel was used with subsequent staining with Coomassie R-250.

Determination of catalytic activity of cytochrome P450scc and its mutant forms. Cholesterol side-chain cleavage activity of cytochrome P450scc and its truncated forms was determined as previously described [18] using HPLC on a C_{18} column (Alltech, 4.6 × 250 mm) with mobile phase of 80% methanol. The amount of progesterone formed from pregnenolone in the presence of cholesterol oxidase was determined at 240 nm. Deoxycorticosterone was used as an internal standard.

RESULTS

Engineering of truncated at C-terminal sequence forms of cytochrome P450scc. Deletion analysis of C-terminal residues, which is a commonly used and informative approach to study the structure–function organization of protein, was successfully used in the present work to understand the functional role of the C-terminal sequence of cytochrome P450scc. To determine the maximal number of amino acid residues that can be removed from the C-terminus of cytochrome P450scc without loss of the ability to correctly bind heme group and without changing electron acceptor properties and activities, amino acids were sequential removed from the C-terminal sequence to reduce the length of the hemeprotein by two, four, seven, and nine amino acids, respectively (Fig. 1, see color insert). In the present work we used the numbering of the amino acid residues for the mature form of cytochrome P450scc according to which arginine Arg472 of the mature form corresponds to residue Arg511 of the precursor of cytochrome P450scc taking into account the N-terminal mitochondrial signal peptide (39 amino acid residues).

As follows from the three dimensional structure of cytochrome P450scc (Fig. 1), the C-terminal fragment is exposed on the surface of the hemeprotein not far from the heme group, is not directly involved in the formation of the heme-binding domain, and is enriched with proline residues and amides of dicarboxylic amino acids.

The truncated forms of cytochrome P450scc engineered in the present work were expressed in *E. coli* cells, purified to an apparent homogeneity, and their functional and catalytic properties were carefully studied. The expression level of deletion mutants of cytochrome P450scc, assessed from the reduced carbon monoxide minus reduced difference spectra, is shown in Fig. 2 (see color insert). Removal of nine (Δ1-9) C-terminal amino

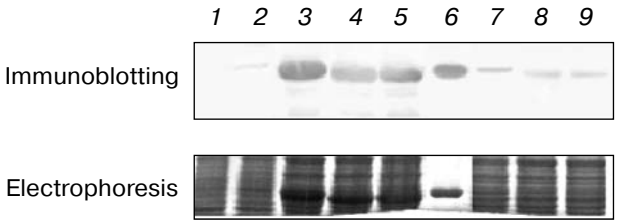

Fig. 3. SDS-PAGE and immunoblotting analysis of cell lysate of recombinant *E. coli* (*1-5*) and solubilized with Emulgen 913 membranes (*6-9*). Immunochemical analysis was carried out using specific antibodies against bovine cytochrome P450scc. Lanes: *1*) lysate of *E. coli* cells; *2*) lysate of *E. coli* transformed with plasmid containing the gene of cytochrome P450scc but without induction; *3*) lysate of *E. coli* transformed with expression plasmid for cytochrome P450scc; *4*, *5*) lysate *E. coli* transformed with expression plasmid for cytochrome P450scc mutant R472Stop; *6*) highly purified cytochrome P450scc; *7*) solubilized membranes of *E. coli* transformed with expression plasmid for cytochrome P450scc; *8*, *9*) solubilized membranes of *E. coli* transformed with expression plasmid for cytochrome P450scc mutant R472Stop.

acid residues of cytochrome P450scc does not result in significant changes of the expression level of the truncated form of cytochrome P450scc as well as the physicochemical and catalytic properties of the hemeprotein. Another interesting point discovered during these experiments is that the expression level of cytochrome P450scc mutants Δ4 and Δ2 is significantly increased (3- and 2-fold, respectively) compared to the wild-type cytochrome P450scc (Fig. 2). Dramatic increase in the expression level of truncated forms appears to be connected with resolution of the possible conformational tension in the C-terminal sequence of the molecule of cytochrome P450scc caused by the presence of two proline residues (Pro481 and Pro482) in wild type hemeprotein, the removal of which facilitates more effective folding during translation of the mutants in *E. coli* cells.

However, the absence of spectrally detected cytochrome P450scc is observed on removal of 10 C-terminal amino acid residues. This fact is not unexpected since Arg472 is a relatively conservative residue (Fig. 1), and removal of the analogous residue in bacterial ortholog CYP51 [10] disturbs the folding of the hemeprotein. In the reduced carbon monoxide minus reduced difference spectrum of the mutant with 10 amino acid residue deletion, there is no absorbance at 450 nm indicating the expression of functionally active hemeprotein, but instead there is absorbance at 420 nm, which is characteristic for denatured form of cytochrome P450. At the same time, SDS-PAGE of cell proteins of recombinant *E. coli* with subsequent immunoblotting analysis indicates the presence of significant amounts of recombinant hemeprotein, which is recognized by antibodies prepared against natural bovine cytochrome P450scc (Fig. 3). Consequently, the removal of 10 amino acid residues from the C-terminal sequence is critical for correct folding and heme binding to the recombinant cytochrome P450scc. This may be connected with the fact that close to Arg472 is located strictly conservative residue Phe471 (Fig. 1), which participates in heme binding, while removal of Arg472 results in disturbance of the conformation of this part of the molecule.

It is interesting that although removal of nine amino acid residues from the C-termini of cytochrome P450scc dramatically decreases the expression level of this mutant, which is three times less compared to wild type protein, we nevertheless were able to purify recombinant cytochrome P450scc Δ9 mutant with the retention of native structure. At the same time, engineering of the Δ7 cytochrome P450scc mutant does not significantly change the expression level of this hemeprotein in *E. coli* cells.

Spectral characterization of truncated forms of cytochrome P450scc. The heterologously expressed truncated forms of cytochrome P450scc, except the mutant Arg472Stop, have been purified to apparent homogeneity as confirmed by SDS-PAGE (Fig. 4). Consequently, the ability of recombinant cytochrome P450scc to correctly fold and interact with immobilized redox-partner (adrenodoxin) does not change upon deletion of up to nine amino acids from the C-terminal sequence, which is confirmed by affinity chromatography on adrenodoxin-Sepharose 4B in the course of purification of the mutants.

The absolute absorbance spectra of the truncated forms are indistinguishable from that of recombinant wild type cytochrome P450scc with the maximum in the Soret region at 393 nm (data not shown). The highly purified truncated forms of cytochrome P450scc are characterized by spectrophotometric index A_{393}/A_{280} equal to 0.75-0.8, which indicates that removal of the C-terminal residues of cytochrome P450scc up to residue 7, does not significantly affect heme binding. The spectra of the reduced

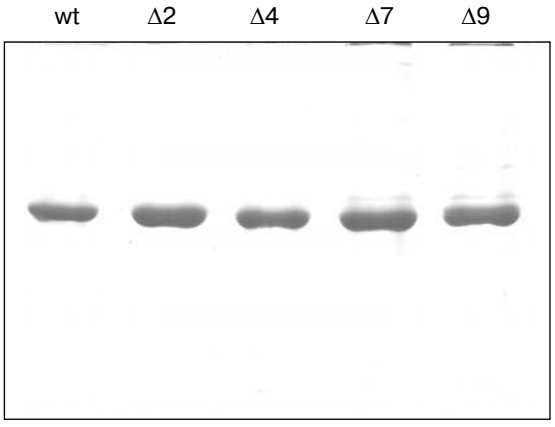

Fig. 4. SDS-PAGE of highly purified C-terminal sequence-truncated forms of cytochrome P450scc. wt, wild type cytochrome P450; Δ2, Δ4, Δ7, and Δ9, cytochromes P450scc truncated by two, four, seven, and nine amino acid residues, respectively.

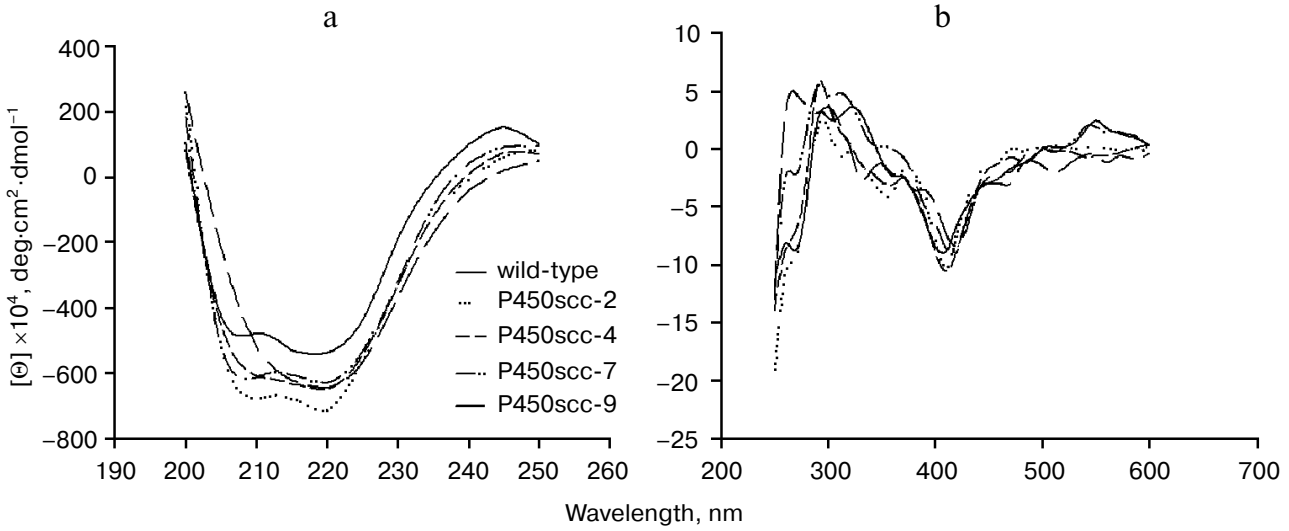

Fig. 5. Circular dichroism spectra of C-terminal sequence-truncated forms of cytochrome P450scc in far-UV (195-250 nm) (a) and visible (300-600 nm) (b) region. The values of optical pathlength and concentration of the sample of cytochrome P450scc were, respectively, 0.1 cm and 1 μM (a) and 0.5 cm and 10 μM (b) in 10 mM potassium phosphate buffer, pH 7.2.

truncated forms in the complex with carbon monoxide confirm the absence of denatured form, cytochrome P420, in the purified hemeproteins.

The possible appearance of structural changes caused by removal of the C-terminal sequence was studied using circular dichroism in the far UV and visible regions. Figure 5 shows circular dichroism spectra of the truncated forms of cytochrome P450scc. In the far UV region (Fig. 5a), the spectra of mutants practically coincide with that of wild type cytochrome P450scc. In the case of removal of nine amino acid residues, the negative maximum of molar ellipticity at 208 nm is less evident, which indicates a decrease in the degree of order of elements of secondary structure. Small differences are also observed in the visible region (Fig. 5b).

Limited proteolysis of truncated forms of cytochrome P450scc. The sensitivity of the cytochrome P450scc molecule to proteolytic modification after removal of C-terminal amino acid residues as well as the probability of the appearance of additional exposed elements was analyzed by using limited proteolysis. As follows from Fig. 6a, the truncated forms of cytochrome P450scc have the same proteolytic picture as the full-length hemeprotein. The cleavage of all analyzed truncated forms of cytochrome P450scc results in the appearance of the two main fragments: F1 (Ile1-Arg250) and F2 (Asn257-the last amino acid), this representing the N- and C-terminal sequences of cytochrome P450scc, respectively [19].

The kinetics of trypsinolysis of full-length and the shortest truncated form (Δ9) of cytochrome P450scc (Fig. 6b) coincide; therefore, the time of trypsinolysis for the other truncated forms was limited to 30 min. It is necessary to stress that the length of fragment F2 of the truncated forms of cytochrome P450scc progressively decreased compared to the full-length form, while the electrophoretic mobility increased, which evidently demonstrates the removal of the C-terminal amino acid sequence of the hemeprotein.

Thus, experiments on limited proteolysis indicate that removal of C-terminal amino acid residues does not result in the appearance of new trypsin-accessible fragments, which may indicate indirectly that the native conformation of cytochrome P450scc is preserved after removal of the C-terminal amino acid sequence.

Catalytic characterization of truncated forms of cytochrome P450scc. Catalytic properties of truncated mutants of cytochrome P450scc after deletion of C-terminal amino acid residues were studied in the reconstituted system containing adrenodoxin reductase and adrenodoxin. Functional activity of cytochrome P450scc and the truncated mutants in the cholesterol side-chain cleavage reaction with formation of pregnenolone differed insignificantly (Fig. 7). This confirms the absence of an effect of peptide Pro473-Ala481 removal on the character of interaction of cytochrome P450scc with substrate and redox-partner – adrenodoxin in the case of the hydroxylation reaction.

DISCUSSION

Despite of wide distribution in nature and multiple substrate specificity, cytochrome P450's have common principles of structural and spatial organization and appear to have a common precursor [20]. This means that individual peculiarities of different forms of cytochrome

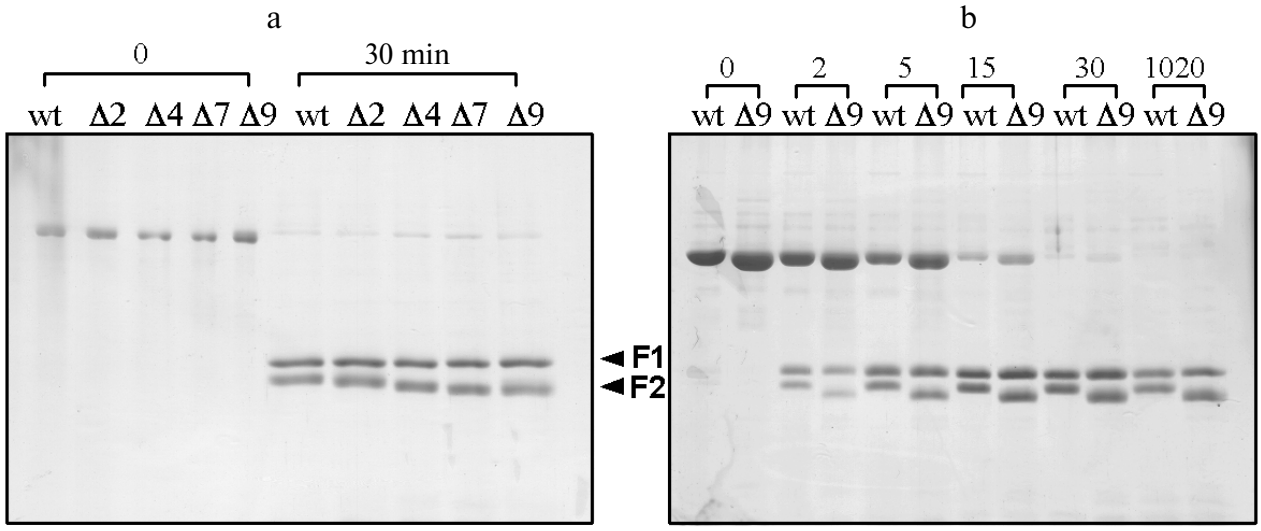

Fig. 6. a) Limited proteolysis of C-terminal sequence-truncated forms of cytochrome P450scc with trypsin: 0, without incubation; 30, after incubation for 30 min. The reaction was carried out at cytochrome P450/trypsin ratio 50 : 1. b) Kinetics of limited trypsinolysis of wild type cytochrome P450scc and deletion mutant Δ9.

P450 are realized at the level of small structural elements and, first of all, structural elements of the active site [21]. One of the most powerful methods to study the molecular organization of proteins in the region of the active site is the preparation of crystals of the proteins of interest with subsequent X-ray analysis. However, the limitations connected with crystallization of membrane-bound cytochrome P450's prevent the preparation of suitable crystals without preliminary protein modification. One of the approaches to overcome this problem is the removal of the N-terminal hydrophobic sequence of cytochrome P450 [22-24]. In the case of cytochrome P450scc which belongs to the group of mitochondrial type cytochrome P450's and does not contain the hydrophobic N-terminal fragment, the removal of the C-terminal sequence allows from one side to decrease the number of hydrophobic amino acid residues and from the other side to elucidate the functional role of this part of the molecule. To determine the number of amino acid residues that could be removed from the C-terminal sequence of cytochrome P450scc without loss of the ability to correctly bind the heme group and correspondently without disturbances of electron-transfer properties, in the present work we sequentially shortened the molecule's length from the C-terminal sequence by two, four, seven, nine, and ten amino acid residues (Fig. 1).

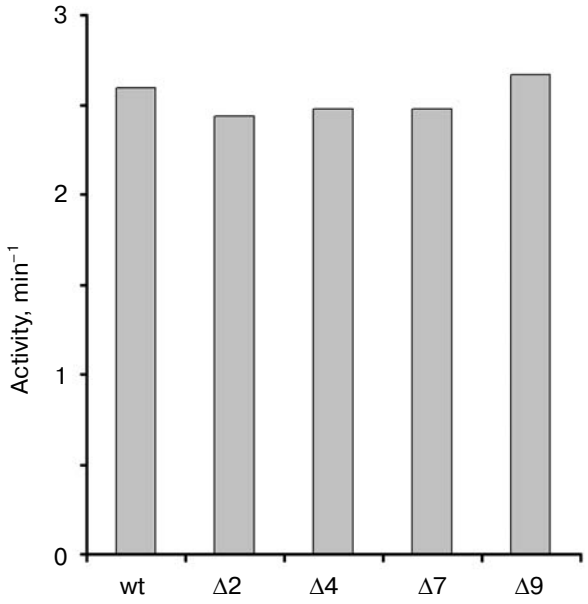

Fig. 7. Catalytic activity of C-terminal sequence-truncated forms of cytochrome P450scc and wild type cytochrome P450scc in cholesterol side-chain cleavage reaction to form pregnenolone in the reconstituted system. Activity is expressed as nanomoles of pregnenolone formed per nanomol of cytochrome P450scc per minute.

The removal of nine C-terminal amino acid residues of cytochrome P450scc does not dramatically change the physicochemical and catalytic properties of the hemeprotein. These truncated mutant forms of cytochrome P450scc retain their ability for correct folding and interaction with adrenodoxin, which allowed the use of affinity chromatography on immobilized ferredoxin for purification of the truncated mutants. An interesting finding is that removal of four amino acid residues from the C-termini of cytochrome P450scc, retaining the functional properties of the hemeprotein, results in an increase (almost 3-fold) in the expression level of the cytochrome

P450scc mutant in *E. coli* (Fig. 2). Optimization of the expression conditions for the Δ4 mutant of cytochrome P450scc increase the expression level up to 4.5 μmol of the recombinant hemeprotein per liter of culture, which is the highest level for heterologous expression of mitochondrial cytochrome P450's at present and opens real promise for structural studies of truncated forms of cytochrome P450scc.

Further modification of the cytochrome P450scc (engineering of the Δ7 mutant) did not significantly affect the expression level. The dramatic increase in expression level after removal of four C-terminal amino acid residues might be connected with the removal of possible conformational tension in the C-terminal fragment of cytochrome P450scc caused by the presence of two proline residues, Pro478-Pro479, in wild type hemeprotein, removal of which facilitates efficient folding of the truncated mutant in *E. coli* cells. This assumption is in accordance with the results of the paper on heterologous expression of cytochrome P45046A1 [22], showing that removal of four proline residues in the C-terminal sequence results in not only increase in the expression level, but also decrease in the aggregation of the hemeprotein.

However, in the reduced carbon monoxide minus reduced difference spectrum of the mutant with deletion of 10 amino acid residues from the C-terminus, there is no 450 nm peak characteristic of functionally active cytochrome P450, but instead there is a peak at 420 nm, which is characteristic for denatured form of cytochrome P450. The absence of the spectrally detected cytochrome P450scc after removal of 10 amino acids was not unexpected since Arg472 (Δ10 mutant) is rather conservative (Fig. 1) and its removal should result in disturbances in hemeprotein folding. At the same time, SDS-PAGE of cellular proteins of recombinant *E. coli* expressing the Δ10 mutant with subsequent immunoblotting analysis indicates the presence of a significant amount of truncated recombinant hemeprotein recognized by antibodies against bovine cytochrome P450scc (Fig. 3). Moreover, specific staining of gels for heme indicates the presence of the proteins containing heme and having molecular weight similar to cytochrome P450scc (data not shown). Consequently, the removal of 10 amino acid residues from the C-termini is critical for correct folding and binding of heme with cytochrome P450scc during its expression in *E. coli*.

An interesting fact discovered in the present work is that although removal of nine amino acid residues significantly decreases expression level of the mutant, which is 3-fold less compared to the wild type hemeprotein, it does not affect the conservation of the native structure as indicated by the characteristic carbon monoxide difference spectrum.

Removal of hydrophobic N-terminal sequence is a well known approach to prepare less hydrophobic forms of cytochrome P450, which is a necessary step in crystallization of the hemeprotein [23-25], but the approach described in the present work consisting of removal of the C-terminal amino acid residues is original and has been practically unused in studies of cytochrome P450. During development of the efficient expression system and purification of microsomal cytochrome P45017α [26], in some cases the attempt to introduce the His-cluster or additional amino acid residues, Ser-Thr-His6, into C-terminal sequence resulted in incorrect folding of cytochrome P45017α.

It was shown earlier that removal of four C-terminal amino acid residues of CYP51 from *Mycobacterium tuberculosis* is followed by the loss of the ability of the hemeprotein to be expressed in *E. coli* in the form of cytochrome P450. However, the removal of 20 amino acid residues from the C-termini of the same isoforms of human or *Candida albicans* cytochrome P450, although it decreases the expression level of spectrally detected cytochrome P450, it does not eliminate its ability to be expressed at all [10]. This indicates that the C-terminal amino acid sequence plays an important role in folding of soluble prokaryotic forms of CYP51 and is not necessary for folding of microsomal eukaryotic sterol-14α-demethylases (*C. albicans* and human). In the present work, it is shown that the C-terminal amino acid sequence of mitochondrial eukaryotic cytochrome P450 plays an important role in folding and heme binding by cytochrome P450scc during its heterologous expression in *E. coli* cells.

Thus, the results presented in the current work indicate that the C-terminal sequence of mitochondrial cytochrome P450scc (Arg472-Ala481) plays an important role in correct folding and heme binding by the recombinant cytochrome P450. The removal of this fragment including critical arginine residue Arg472 results in loss of ability for correct folding and heme binding, as indicated by the absence of spectral parameters characteristic for the hemeprotein. On the basis of these results and the literature data [10], we suggest that the folding of mitochondrial cytochrome P450scc during its heterologous expression in *E. coli* bacterial cells is realized through mechanisms more closely related to folding of soluble bacterial cytochrome P450's, rather than microsomal eukaryotic cytochrome P450's.

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       520          *          540          *
MTCYP51     : HSKMVVQLAQPACVRY     TGV----------  : 451
11A1bovine  : IFNLILTPDKPIFLVF   PFNQDPPQA ---------  : 520
11A1human   : TFNLILMPEKPISFTFWPFNQEATQQ   ---------  : 521
11A1sheep   : IFNLILTPDKPIFLVF   PFNQGPPQA ---------  : 520
11A1goat    : IFNLILTPDKPIFLVF   PFNQDPPQA ---------  : 520
11A1pig     : IFNLILMPDKPIFLVF   PFNQDPLQA ---------  : 520
11A1rat     : KFNLILMPEKPIFFNFQPL   QDLGSTMPRKGDTV -  : 526
11A1R.trou  : TFELILLPEKPILLTL   PLKSGQ ------------  : 514
11B1human   : VYSFILRPSMCPLLTF   AIN----------------  : 503
11B1bovine  : VYRFILMPSTLPLFTF   AIQ----------------  : 503
11B1hamste  : VYRFVLAPSSSPLLTF   PVS----------------  : 499
11B1pig     : IYRFIMTPSTLPLLTF   AIS----------------  : 503
11B1rat     : VFRFLLMPSSSPFLTF   PVS----------------  : 499
11B1frog    : VFGFILMPEKPPLLTF   PI-----------------  : 517
11B2human   : VYSFILRPGTSPLLTF   AIN----------------  : 503
11B2rat     : AYRFVLMPSSSPVLTF   PIS----------------  : 510
11B2hamste  : VYRFVLAPSSSPLLTF   PVS----------------  : 500
11B3rat     : AYRFVLMPSSSPVLTF   PIS----------------  : 500
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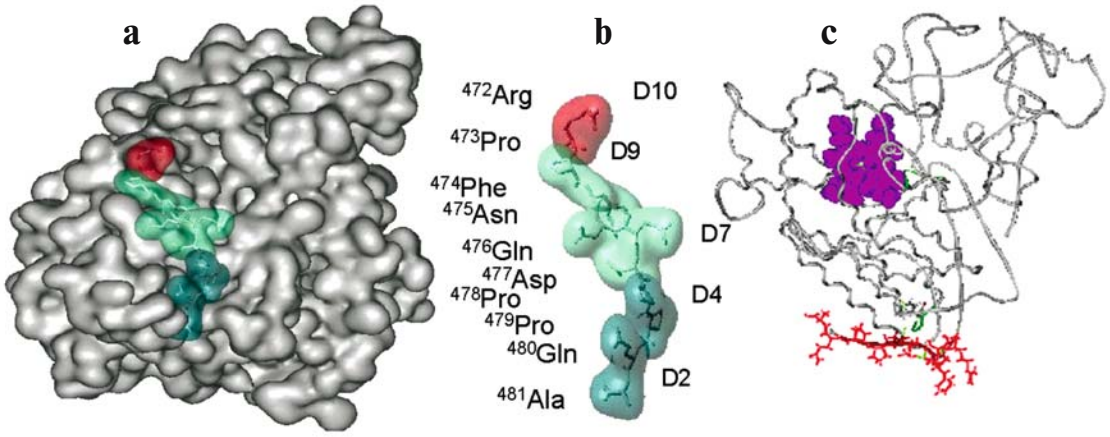

Fig. 1. (N. V. Strushkevich et al.) Alignment of the C-terminal sequences of mitochondrial isoforms of cytochrome P450 and bacterial cytochrome P450 from *M. tuberculosis* (CYP51). Location of the C-terminal amino acid sequence of cytochrome P450scc in the tertiary structure [27]. The sequences were taken from PubMed. Location of C-terminal fragment Arg472-Ala481 on the surface of cytochrome P450scc molecule (a), its spatial organization (b), and disposition in tertiary structure of cytochrome P450scc (c).

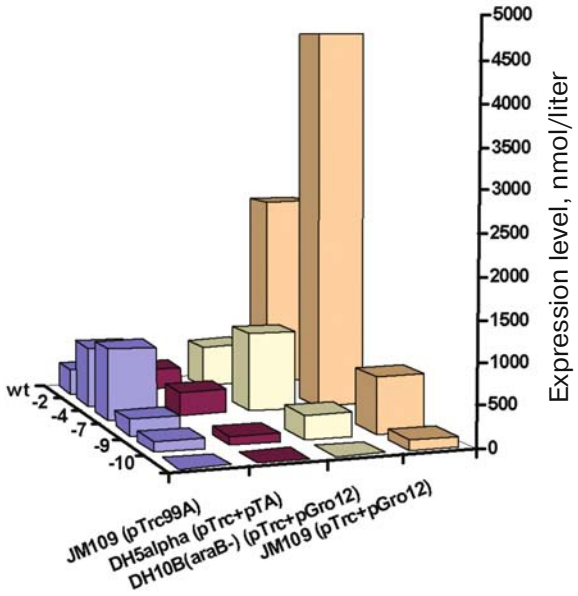

Fig. 2. (N. V. Strushkevich et al.) Level of heterologous expression of truncated forms of cytochrome P450scc in *E. coli*. The content of cytochrome P450scc was assessed from the reduced carbon monoxide minus reduced difference spectra of cytochrome P450 in nanomoles of recombinant cytochrome P450 per liter of culture. The expression of the wild type cytochrome P450scc is taken as 100%; wt, wild-type cytochrome P450.