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Site-Directed Mutagenesis of Cytochrome P450scc. II. Effect of Replacement of the Arg425 and Arg426 Residues on the Structural and Functional Properties of the Cytochrome P450scc

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Abstract—Cytochrome P450-dependent monooxygenases, in spite of their wide distribution, can be simply divided into a few groups differing in the location of the electron transfer chain and their composition. The two main groups of cytochrome P450-dependent monooxygenases are the mitochondrial and microsomal enzymes. While in two-component microsomal cytochrome P450-dependent monooxygenases electrons are supplied to cytochrome P450 by a flavoprotein (NADPH-cytochrome P450 reductase), in three-component mitochondrial monooxygenases the electrons are supplied to cytochrome P450 by a low molecular weight protein (ferredoxin). The interaction of cytochrome P450 with NADPH-cytochrome P450 reductase and ferredoxin is the subject of intensive studies. Using chemical modification, chemical cross-linking, and site-directed mutagenesis, we identified surface exposed positively charged residues of cytochrome P450scc which might be important for interaction with adrenodoxin. Theoretical analysis of the distribution of surface electrostatic potential in cytochrome P450 indicates that in contrast to microsomal monooxygenases, cytochromes P450 of mitochondrial type, and cholesterol side-chain cleavage cytochrome P450 (P450scc) in part, carry on the proximal surface an evidently positively charged site that is formed by residues Arg425 and Arg426. In the present work, to estimate the functional role of Arg425 and Arg426 of cytochrome P450scc, we used site-directed mutagenesis to replace these residues with glutamine. The results indicate that residues Arg425 and Arg426 are involved in the formation of a heme-binding center and electrostatic interaction of cytochrome P450scc with its physiological electron-transfer partner, adrenodoxin.

Key words: cytochrome P450scc, site-directed mutagenesis, heterologous expression, protein–protein interactions

Cholesterol side-chain cleavage cytochrome P450 (P450scc, CYP11A1) of the inner mitochondrial membrane of adrenal cortex catalyzes three sequential reactions of cholesterol oxidation resulting in cleavage of the cholesterol side chain and formation of pregnenolone, the precursor of the main steroid hormones [1]. These reactions include cholesterol hydroxylation at position 22 with formation of 22R-hydroxycholesterol, the subsequent hydroxylation of 22R-hydroxycholesterol at position 20 to form 20R,22R-dihydroxycholesterol, and finally the 20,22-lyase reaction with cleavage of the cholesterol side chain and formation of pregnenolone. The conversion of cholesterol to pregnenolone is the key and rate-limiting step of steroid hormone biosynthesis and is controlled by a number of regulatory mechanisms. P450scc receives the electrons necessary to activate molecular oxygen from NADPH via a short electron transfer chain consisting of a flavoprotein (adrenodoxin reductase) and a ferredoxin (adrenodoxin). To convert cholesterol to pregnenolone, P450scc should receive from adrenodoxin at least six electrons. The intrinsic mechanism of electron transfer from NADPH to P450scc via adrenodoxin reductase and adrenodoxin is not clear, but it is evident that in the process of electron transfer adrenodoxin reductase, adrenodoxin, and P450scc form highly specific complexes in which an electron is transferred from one prosthetic group to another. The catalytic cycle of P450scc starts from cholesterol binding and receiving the first electron from adrenodoxin.

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The interaction of P450scc with adrenodoxin has been studied intensively [2-12]. The sensitivity of the interaction of cytochrome P450scc with adrenodoxin to changes in ionic strength indicates the possible involvement of electrostatic interactions in this interaction. This, however, does not exclude the participation of hydrophobic interactions. The involvement of positively charged residues of cytochrome P450scc in the interaction with adrenodoxin was proved by chemical modification experiments. Thus, modification of surface-exposed lysine residues of cytochrome P450scc prevents formation of the affinity complex with adrenodoxin [3-7]. Site-directed mutagenesis of lysine Lys338 and Lys342 (nomenclature of the mature form of cytochrome P450scc) increases the dissociation constant of the complex of cytochrome P450scc with adrenodoxin more than 100-fold, indicating the involvement of these resides in the interaction [13]. Besides lysine Lys338 and Lys342, located in α-helix K of cytochrome P450scc, the positively charged lysine residues Lys403 and Lys405, located in the so-called "meander", are also extremely important for electrostatic interactions of cytochrome P450scc with adrenodoxin [14].

On the other hand, chemical modification and site-directed mutagenesis of negatively charged residues of adrenodoxin support their involvement in electrostatic interaction with cytochrome P450scc [9, 15-17].

Thus, the available literature data as well as our own results indicate the important role of electrostatic interactions between positively charged residues of cytochrome P450scc and negatively charged residues of adrenodoxin in formation of an affinity complex between the two proteins in the frame of which an electron is transferred from adrenodoxin to cytochrome P450scc. However, the questions of the sizes of the interacting surfaces and the specific amino acid residues involved in the formation of the complex are still not clear.

The aim of the present work was to elucidate the functional role of the Arg425 and Arg426 residues, which are located on the proximal surface of cytochrome P450scc, in stabilization of the structure of cytochrome P450scc. The choice of residues Arg425 and Arg426 of cytochrome P450scc for site-directed mutagenesis is based on the theoretical analysis of the distribution of electrostatic potential on the proximal surface of cytochrome P450scc which, as has been shown earlier, is responsible for the interaction with protein partners [18].

The data presented in this paper indicate that the selective replacement of Arg425 and Arg426, located on the proximal surface of cytochrome P450scc, results in dramatic changes in the structure and function of the protein. Thus, replacement of Arg425 of cytochrome P450scc with glutamine results in the loss of the ability of apo-cytochrome P450scc to interact in the correct way with the heme group, while the replacement of Arg426 with glutamine results in complete loss of the ability of cytochrome P450scc to interact with adrenodoxin. The data indicate that Arg426 of cytochrome P450scc is involved in the electrostatic interaction of the cytochrome with adrenodoxin.

MATERIALS AND METHODS

In the present work we used isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Gibco BRL, USA), yeast extract, peptone, tryptone (Difco, USA), cholesterol, pregnenolone, sodium cholate, Tween-20, Coomassie G-250, glucoso-6-phosphate, glucoso-6-phosphate dehydrogenase, polyethylene glycol (6 kD), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (Serva, Germany), Sepharose 4B, CNBr-activated Sepharose 4B, DEAE-Sepharose 6B (Pharmacia, Sweden), δ-aminolevulinic acid, phenylmethylsulfonyl fluoride (PMSF), NADP(H^+) (Sigma, USA), TSK-gel HW-50 (Toyopearl, Japan), and Bio-Gel HTP (Bio-Rad, USA).

Site-directed mutagenesis of cytochrome P450scc. Site-directed mutagenesis of cytochrome P450scc was performed using the Quick Change reagent kit (Stratagene, USA) with the following mutagenic primers:

R425Q G TGC GTG GGC CAG CGG ATC GCC (SENCE)
GGC GAT CCG CTG GCC CAC GCA C (ANTISENSE)

R426Q GC GTG GGC CGG CAG ATC GCC GAG C
G CTC GGC GAT CTG CCG GCC CAC GC

R425Q,R426Q G TGC GTG GGC CAG CAG ATC GCC GAG CTG
C CAG CTC GGC GAT CTG CTG GCC CAC GCA

R425Q,R426C G TGC GTG GGC CAG TGC ATC GCC GAG CTG
C CAG CTC GGC GAT GCA CTG GCC CAC GCA

The presence of a desired substitution in cDNA coding cytochrome P450scc was confirmed by automatic sequencing on an A377 DNA sequencer (Applied Biosystems, USA).

Expression and purification of recombinant proteins. Recombinant proteins of the cytochrome P450-dependent monooxygenase system from bovine adrenocortical mitochondria (cytochrome P450scc, adrenodoxin, and adrenodoxin-reductase) were expressed in *E. coli* cells. The plasmid (pTrc99A) containing cDNA encoding the mature form of cytochrome P450scc was kindly provided by Prof. M. R. Waterman (Vanderbilt University, USA). The plasmid containing cDNA encoding mature adrenodoxin (pBa1159) and adrenodoxin-reductase (pBAR1607) were kindly provided by Prof. A. Sagara (Kochi Medical School, Japan).

Recombinant adrenodoxin reductase and adrenodoxin were purified as previously described [14, 19]. The

highly purified recombinant adrenodoxin and adrenodoxin reductase have spectrophotometric indexes $A_{271}/A_{450} = 7.2$ and $A_{414}/A_{280} = 0.94$, respectively.

Wild-type and mutant forms of cytochrome P450scc were isolated and purified according to a scheme developed for purification of the substrate-bound high-spin form of the cytochrome [20, 21]. The scheme includes sequential ultrasonic treatment of *E. coli* cells, solubilization of the sonicated lysate with Emulgen 913, precipitation of the solubilized cytochrome P450scc with polyethylene glycol (6 kD) at final concentration 20%, incubation of solubilized pellet with cholesterol, and affinity chromatography of the cytochrome P450scc on adrenodoxin-Sepharose 4B.

Analytical methods. The protein content of recombinant bacterial cells after expression and the purity of final preparations were analyzed using SDS-PAGE in 12% gel according to Laemmli [22] using a mini Protean II apparatus (Bio-Rad, USA). Immunochemical identification of recombinant proteins was performed by immunoblotting [23]. The concentrations of cytochrome P450scc, adrenodoxin-reductase and adrenodoxin were determined using 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, respectively [24, 25].

Spectral characterization of cytochrome P450scc mutants. Spectrophotometric measurements were done using a Shimadzu UV-3000 (Shimadzu, Japan) spectrophotometer. The concentration of cytochrome P450scc and its denatured form (cytochrome P420) was determined from carbon monoxide difference spectra of the dithionite-reduced cytochromes using molar extinction coefficients 91 $mM^{-1}\cdot cm^{-1}$ at 450 nm and 114 $mM^{-1}\cdot cm^{-1}$ at 420 nm according to the equation given in [26]. The records of reduced carbon monoxide difference spectra were also used to assess the expression level of cytochrome P450scc in *E. coli* cells and to determine cytochrome P450scc concentration at intermediate steps of its isolation and purification. The content of the high-spin form of cytochrome P450scc in the purified cytochrome P450scc was calculated from absolute absorbance spectra using the ratio $\Delta A_{390\text{-}470}/\Delta A_{416\text{-}470} = 0.4$ and 2.2 for low- and high-spin forms of the cytochrome, respectively [27]. The ratios of peak intensities (tyrosine/tryptophan) $\Delta A_{287\text{-}288.3}/\Delta A_{295\text{-}290.5}$ in the second derivatives of the ultraviolet spectra of cytochrome P450scc were determined according to [28].

Enzymatic reduction of cytochrome P450scc. The rate of enzymatic reduction of cytochrome P450scc in a reconstituted system containing adrenodoxin reductase and adrenodoxin (ratio of adrenodoxin reductase/adrenodoxin/cytochrome P450scc = 0.5 : 2 : 1 (M/M)) was calculated from carbon-monoxide difference spectra of enzymatically or chemically reduced cytochrome P450scc [29].

Determination of cholesterol side-chain cleavage activity of cytochrome P450scc and its mutant. Cholesterol side-chain cleavage activity of cytochrome P450scc and its Arg426 mutant form was analyzed according to [30] by HPLC on a 4.6 × 250-mm Zorbax-Sil column with the system hexane–isopropanol (3 : 1). The amount of progesterone formed from the reaction product (pregnenolone) was determined at 240 nm in the presence of cholesterol oxidase. Deoxycorticosterone was used as an internal standard.

Monitoring of spin changes in cytochrome P450scc during its interaction with adrenodoxin. Spectral changes in cytochrome P450scc induced by adrenodoxin were recorded as previously described [31]. The apparent dissociation constants for the cytochrome P450–adrenodoxin complex were determined in 20 mM HEPES-buffer, pH 7.2, containing 0.1% Tween-20, 0.1 mM EDTA, 0.1 mM DTT, 50 mM sodium chloride, and 20 μM cholesterol [8]. The concentration of cytochrome P450scc in the sample was 1 μM. The change in the optical density $\Delta A_{100} - \Delta\varepsilon_{390\text{-}420} = 110$ $mM^{-1}\cdot cm^{-1}$ was taken as 100% change of the spin state [32]. The absence of denatured cytochrome P450scc in the sample was shown by recording the reduced carbon monoxide difference spectrum.

RESULTS

One of the characteristic features of cytochrome P450-dependent monooxygenation is its unique ability to activate molecular oxygen such that it can insert one activated oxygen species into the molecule of the oxidizing organic compound independent of its chemical structure. To activate molecular oxygen, cytochrome P450 should sequentially transfer two electrons to it, which the cytochrome in turn receives from its electron transfer partners. Depending on the location and content of the electron transfer chain, all cytochrome P450-dependent monooxygenases are subdivided into several groups. The two main groups are the microsomal and mitochondrial monooxygenases. Mitochondrial type cytochromes P450, being localized mostly in the inner mitochondrial membrane but also found in some bacteria, receive electrons from a low-molecular-weight negatively charged intermediate electron transfer protein, a Fe_2S_2-type ferredoxin. In contrast, cytochromes P450 of microsomal type, predominantly localized in endoplasmic reticulum as well as in some microorganisms, receive electrons from a flavoprotein, NADPH-cytochrome P450 reductase. In the process of electron transfer from NAD(P)H to cytochrome P450, mitochondrial and microsomal cytochromes P450 form specific complexes with ferredoxin and flavoprotein, respectively, within which electrons are transferred to cytochrome P450 and then to molecular oxygen. Previous studies of the interaction of electron transfer partners in cytochrome P450-dependent monooxygenases have shown that electrostatic interactions play an extremely important role in these interactions.

The mechanism of the interaction of cytochrome P450 with its electron transfer partners is of great importance but still not well understood. The studies of this interaction using chemical modification, chemical cross-linking, and site-directed mutagenesis confirmed the participation of the charged amino acid residues of electron transfer proteins in this interaction. The sensitivity of this interaction to the ionic strength confirms the important role of electrostatic interactions in complex formation.

The presence of a negatively charged cluster including residues Asp79, Asp76, Glu74, Glu73, and Asp72 in adrenodoxin has been shown by chemical modification and site-directed mutagenesis [9, 15-17]. This indicates a possible involvement of the positively charged residues of cytochrome P450scc in interaction with the ferredoxin.

The crystal structure of truncated [33] and full-length adrenodoxin [34] confirms the presence of a negatively charged interaction domain in the ferredoxin. This negatively charged helical domain includes residues Asp79, Asp76, Asp72, Glu74, and Glu73, which were previously mapped as candidates for electrostatic interaction with adrenodoxin reductase and cytochrome P450scc [9, 15-17].

Previous studies on the interaction of cytochrome P450scc with adrenodoxin using chemical modification [5-8], chemical cross-linking [11], immunochemical analysis [35, 36], and site-directed mutagenesis [13, 14] mapped some positively charged residues (mostly lysines) of cytochrome P450scc that might be involved in interaction with adrenodoxin. The alignment of amino acid sequences of cytochrome P450scc with the sequences of other cytochromes P450 of known crystal structure indicates that these residues are distributed in the following structural elements of cytochrome P450scc: Lys109 and Lys110, α-helix C; Lys338 and Lys342, α-helix K; Lys403 and Lys405, the "meander" on the proximal surface of cytochrome P450scc, close to the heme-binding peptide. This peptide is supposed to be involved in the interaction between the cytochrome and its electron transfer partners [18, 37].

Analysis of the interaction of cytochrome P450scc with adrenodoxin raises the question whether the exposed positively charged residues of cytochrome P450scc that have been mapped before are sufficient for the interaction between cytochrome P450scc and adrenodoxin and, if not, how many additional residues of the cytochrome are involved in the interaction. Comparison of the distribution of electrostatic potential on the proximal surface of mitochondrial and microsomal cytochromes P450, which is thought to be the main docking site with electron transfer proteins [18, 34], indicates that mitochondrial cytochrome P450, in contrast to microsomal heme proteins, have an extra positively charged site close to the heme-binding peptide (Fig. 1). This positively charged element of the cytochrome P450 structure is located very close to the heme group from the proximal side, and it can be envisaged as an excellent candidate for the site responsible for the interaction with adrenodoxin. In cytochrome P450scc, this fragment is represented by the sequence just after the Cys422 residue, which serves as a

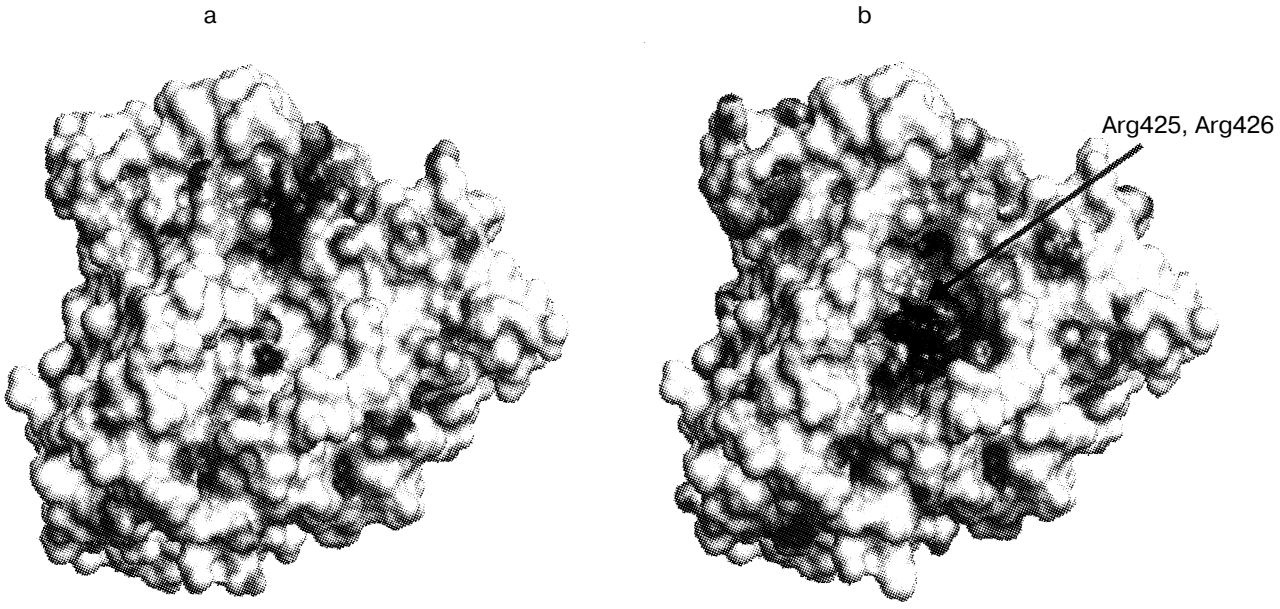


Fig. 1. Distribution of electrostatic potential of the proximal surface of cytochrome P450 of microsomal (a) and mitochondrial type (b) with indication of positively charged residues (Arg425 and Arg426 in cytochrome P450scc) that appear to be involved in the interaction of cytochrome P450scc with the ferredoxin.

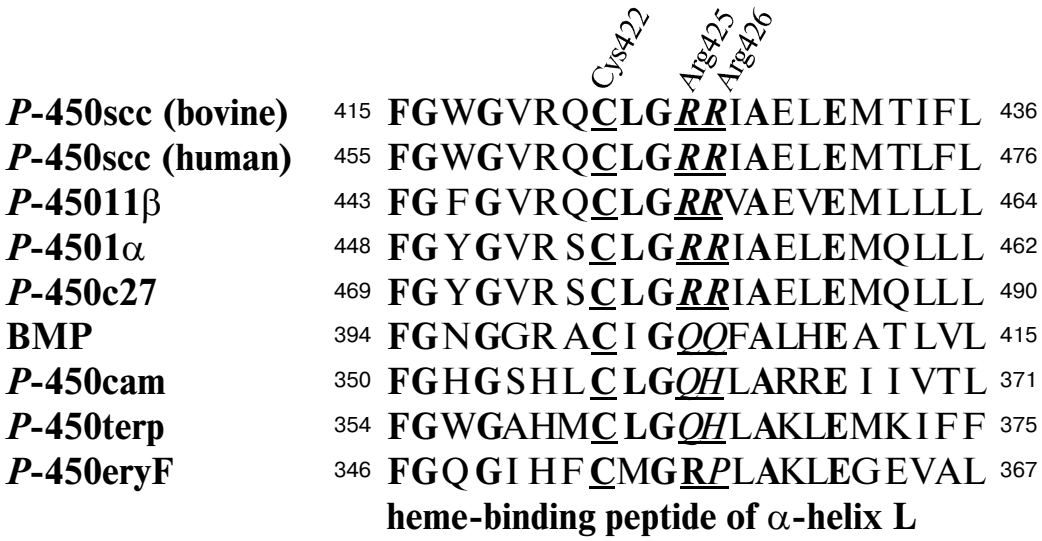


Fig. 2. Alignment of amino acid sequences of different cytochromes P450 of mitochondrial and microsomal types in the region of the heme-binding peptide, which includes the Arg425 and Arg426 residues of cytochrome P450scc. Amino acid sequences were downloaded from PubMed. Numbering for cytochrome P450scc is indicated as for the mature form.

proximal ligand of cytochrome P450scc. This sequence includes positively charged residues Arg425 and Arg426.

Figure 2 shows the alignment of the amino acid sequence of cytochrome P450scc with the sequence of mitochondrial and microsomal cytochromes P450 in the heme-binding region. As follows from this alignment, the Arg425 and Arg426 residues are rather conservative among mitochondrial cytochromes P450, suggesting that these residues might be important for catalysis. It is of interest that in cytochrome P450cam, a bacterial representative of mitochondrial cytochromes P450, two homologous arginine residues are four residues shifted toward the C-terminus. Cytochrome P450eryf contains only one arginine residue homologous to Arg425 of cytochrome P450scc, while microsomal cytochrome P4503A4 has an arginine residue homologous to Arg426 of cytochrome P450scc. It is interesting that in cytochrome P450BM3, which is usually used for molecular modeling of microsomal-type cytochromes P450 and has been used to model the 3-dimensional structure of cytochrome P450scc [38, 39], amino acid residues homologous to Arg425 and Arg426 are represented by the Gln403 and Gln404 residues.

To understand the functional role of residues Arg425 and Arg426 of cytochrome P450scc, in the present work we used site-directed mutagenesis to replace these residues sequentially and individually with the glutamine residues (Gln) present in cytochrome P450BM3. We choose cytochrome P450BM3 because it was used as a prototype to build the model of 3-dimensional structure of cytochrome P450scc by molecular modeling [38, 39].

Figure 3 shows experimental data on the heterologous expression and distribution between cytosolic and membrane fractions of recombinant *E. coli* cells of the mutant forms of cytochrome P450scc: Arg425Gln, Arg426Gln, Arg425GlnArg426Gln, and Arg425GlnArg426Cys. The inset shows the results of immunoblotting. Spectrally

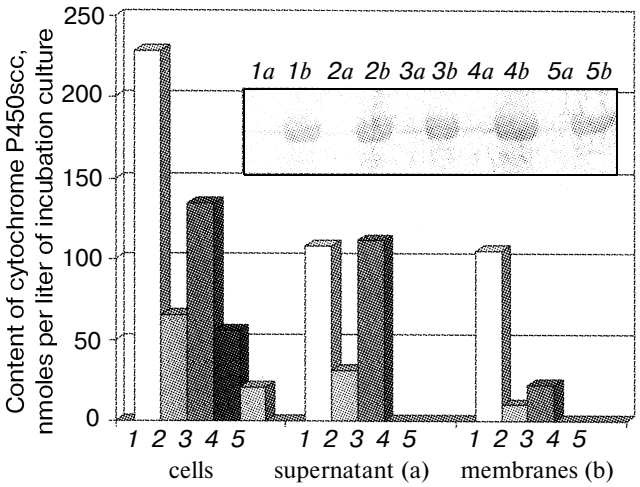


Fig. 3. Spectrophotometrically detectable expression level of wild-type and cytochrome P450scc forms in *E. coli* cells: *1*) wild-type cytochrome P450scc; *2*) Arg425Gln; *3*) Arg426Gln; *4*) Arg425GlnArg426Gln; *5*) Arg425GlnArg426Cys. The distribution of cytochrome P450scc mutant forms between subcellular fractions of *E. coli* cells. Inset: immunoblotting of soluble (a) and membrane (b) fractions of recombinant *E. coli* cells in the presence of antibodies against cytochrome P450scc.

Table 1. Isolation and purification of Arg426Gln mutant form of cytochrome P450scc from recombinant *E. coli* cells

Purification step	Spectrally detectable cytochrome P450scc, nmole	Yield, %
Bacterial culture	485	100
Sonicated cells	388	80
Solubilizate	340	69
Pellet after PEG	255	53
Ad-Sepharose	*	*
Hydroxyapatite	116	24
Cholate-Sepharose	60	12

* Does not interact with adrenodoxin-Sepharose 4B.

detected expression levels of mutant forms of cytochrome P450scc are considerably lower compared to the expression of wild-type cytochrome P450scc indicating the important structural role of these residues. The replacement of Arg425 and the double substitution of Arg425 and Arg426 were critical for cytochrome P450scc. In the later case, the cytochrome was practically not detected by spectral analysis of the recombinant cells. However, SDS-PAGE and immunoblotting analysis of cytosolic and membrane fractions of recombinant *E. coli* indicated the presence of significant amounts of the protein recognized with antibodies against cytochrome P450scc. Thus, the replacement of Arg425 results in serious changes of the protein folding and heme insertion followed by loss of ability of cytochrome P450scc to bind heme and acquire the spectral properties characteristic for this heme protein. This is not surprising since Arg425 is located close to cysteine Cys422, which serves as the proximal ligand of cytochrome P450scc. The loss of the ability of cytochrome P450scc to coordinate heme after replacement of Arg425 appears to be connected with disturbance of the conformation of the heme-binding fragment (the N-terminal part of α-helix L) since this residue is very close to the fifth ligand of cytochrome P450scc, Cys422.

However, site-directed replacement of arginine Arg426 for Gln, which significantly decreased the expression level of the mutant, retained the possibility for isolation and purification of the mutant form for physicochemical characterization and study of the functional role of Arg426. Table 1 summarizes results on isolation and purification of the Arg426Gln mutant of cytochrome P450scc from recombinant *E. coli* cells. The main problem we met during purification of this mutant form of the cytochrome was the inability of the mutant to interact with immobilized adrenodoxin, which is usually used as an affinity matrix for purification of wild-type cytochrome P450scc [14, 21]. Consequently, supporting our suggestion made based on theoretical analysis of electrostatic potential distribution on the proximal surface of cytochrome P450scc, this fact indicates that the Arg426 residue is indeed involved in electrostatic interaction with adrenodoxin.

The replacement of affinity chromatography on immobilized adrenodoxin with hydrophobic chromatography on cholate-Sepharose 4B (immobilized cholic acid) allowed us to isolate and purify the Arg426Gln mutant form of cytochrome P450scc in homogeneous state. The highly purified Arg426Gln mutant is in a native form based on its maximum at 450 nm in the absorption spectrum of dithionite reduced carbon monoxide complex and does not contain denatured protein (the absence of significant absorbance at 420 nm). The absolute absorption spectra of the Arg426Gln mutant form in the oxidized state and in complex with cholesterol in comparison with the same spectra of wild-type cytochrome P450scc are presented in the Fig. 4. Curve *2* in Fig. 4 shows that the Arg426Gln mutant is predominantly in the high-spin form, which is explained by the use of cholesterol in the buffer solution to stabilize the cytochrome P450scc during purification. This also indicates that

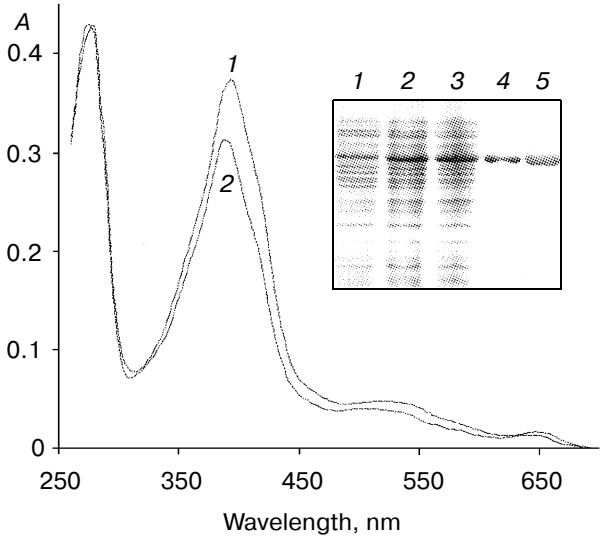


Fig. 4. Absolute absorption spectra of (*1*) recombinant wild-type cytochrome P450scc and (*2*) the Arg426Gln mutant form of cytochrome P450scc in 50 mM potassium phosphate buffer (pH 7.4) containing 1 M NaCl, 0.3% sodium cholate, and 0.1 mM EDTA. Concentration of cytochrome P450scc, 2 μM. Inset: SDS-PAGE of the proteins of recombinant *E. coli* cells (*1*), Arg425Gln (*2*), Arg426Gln (*3*), and highly purified cytochrome P450scc (*4*) and the Arg426Gln mutant form of cytochrome P450scc (*5*).

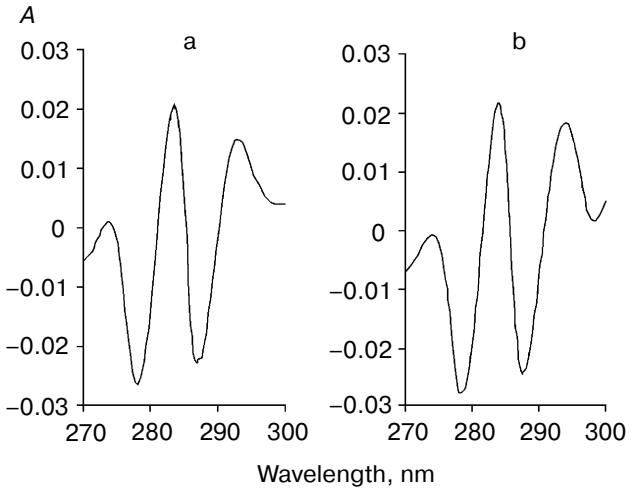


Fig. 5. Second derivative of ultraviolet absorption spectra of recombinant wild-type (a) and Arg426Gln mutant (b) forms of cytochrome P450scc in 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA. Concentration of cytochrome P450scc, 2 μM.

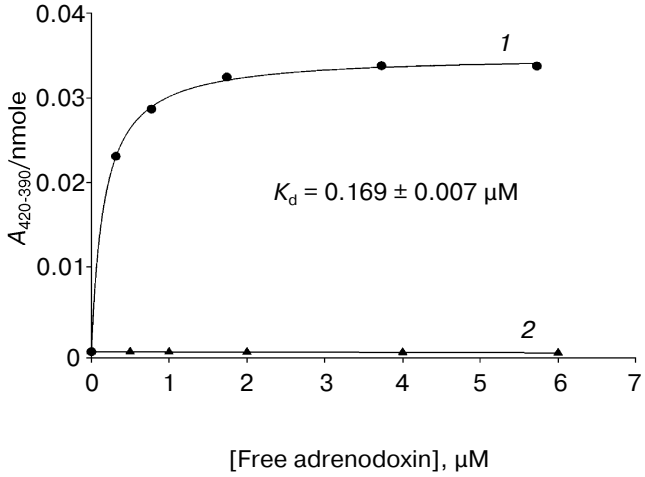


Fig. 6. Dependence of the spectral changes $A_{420\text{-}390}$ reflecting spin change from the low- to the high-spin form for cytochrome P450scc (*1*) and the Arg426Gln mutant (*2*) on the concentration of adrenodoxin added.

replacement of Arg426 for Gln does not affect the ability of the Arg426Gln mutant to interact with cholesterol.

The absence of major differences between the second derivatives of the ultraviolet spectra of the Arg426Gln mutant and wild-type cytochrome P450scc (Fig. 5), which are very sensitive to changes in protein conformation [28] and reflect the average polarity of the microenvironment of the tyrosine and tryptophan residues, supports our conclusion that there are no major changes in the folding of the Arg426Gln mutant form.

The spectral properties of the highly purified Arg426Gln mutant form of cytochrome P450scc, the homogeneity of which is supported by SDS-PAGE (Fig. 4), are summarized in Table 2.

Thus, the data confirm our suggestion that site-directed mutagenesis of Arg426 of cytochrome P450scc, which is located in the region of the heme-binding peptide close to Cys422 (a proximal ligand of cytochrome P450scc), does not result in evident changes in the folding and content of the structural elements of cytochrome P450scc. This indicates that the inability of the Arg426Gln mutant form of cytochrome P450scc to interact with immobilized adrenodoxin is only due to disturbance in interaction of the two proteins.

To further prove the inability of the Arg426Gln mutant form of cytochrome P450scc to interact with adrenodoxin, we carried out spectrophotometric titration experiments of the cytochrome P450scc mutant with adrenodoxin. The addition of adrenodoxin to the low-spin form of the wild-type cytochrome P450 causes spectral changes reflecting spin shift from the low- to high-spin form (Fig. 6). The dissociation constant of the cytochrome–adrenodoxin complex determined from the dependence of the amplitude of the spectral changes on the concentration of free adrenodoxin is 0.169 ± 0.007 μM. However, addition of adrenodoxin in the same range of concentrations to the Arg426Gln mutant does not cause any spectral changes in the cytochrome, indicating that the replacement of Arg426 for Gln results in complete loss of the ability of cytochrome P450scc to either physically interact with immobilized adrenodoxin or to respond on interaction by changing of the spin state.

Table 2. Comparison of spectral characteristics of highly purified Arg426Gln mutant and wild-type forms of cytochrome P450scc

P450scc	C, μM	$I_{393/280}$	$A_{393\text{-}470}/A_{416\text{-}470}$	High spin form, %	$A_{287\text{-}283}/A_{295\text{-}290.3}$	C_{CO}/C_{abs}*
Wild type	225.0	0.88	1.51	69	1.23	67
Arg426Gln	35.5	0.75	1.74	79	1.18	65

* The ratio of concentrations of cytochrome P450scc and Arg426Gln mutant form determined from the difference spectra of carbonyl complex of the reduced form of cytochrome P450scc and absolute absorption spectrum.

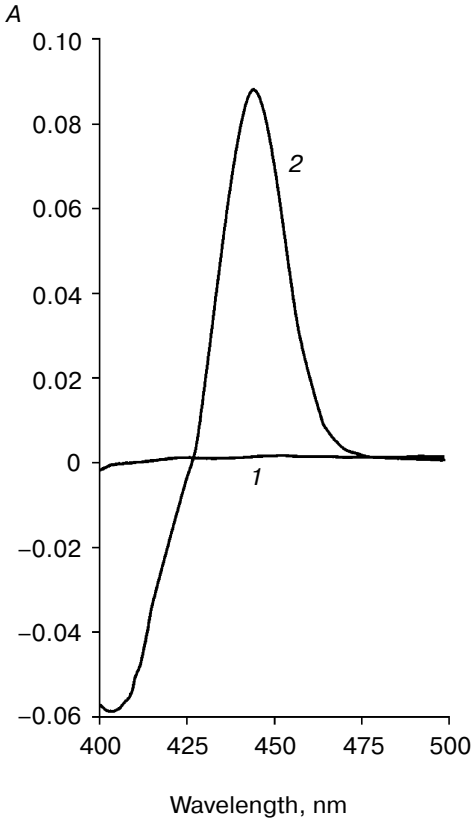


Fig. 7. Carbon monoxide difference spectra of Arg426Gln mutant form of cytochrome P450scc under conditions for enzymatic reduction in the reconstituted system containing adrenodoxin reductase and adrenodoxin at ratio 1 : 2 (*1*) and chemically reduced with sodium dithionite (*2*). Concentration of the mutant cytochrome, 1 μM.

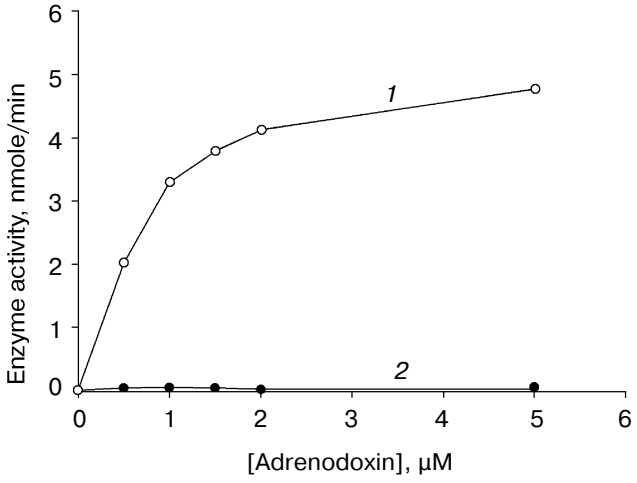


Fig. 8. Dependence of catalytic activity of wild-type (*1*) and Arg426Gln mutant cytochrome P450scc (*2*) in the cholesterol side-chain cleavage reaction on adrenodoxin concentration.

Finally, to further demonstrate the inability the Arg426Gln mutant of cytochrome P450scc to interact with adrenodoxin, we did experiments on chemical and enzymatic (in the presence of adrenodoxin reductase and adrenodoxin) reduction of the Arg426Gln mutant in the presence of carbon monoxide (Fig. 7). In agreement with earlier data on the inability of the Arg426Gln mutant to interact with immobilized adrenodoxin, the data presented in Fig. 7 confirm the inability of reduced adrenodoxin to transfer electrons to the cytochrome P450 mutant. However, the Arg426Gln mutant is effectively reduced by sodium dithionite, showing that this mutant is not a denatured form of cytochrome P450scc. Rather, the replacement of Arg426 results in the loss of the ability of the mutant to accept electrons from the physiological partner—adrenodoxin.

The main biological role of cytochrome P450scc is oxidative cleavage of the side chain of cholesterol to form pregnenolone, the precursor of all of the main steroid hormones. To evaluate the effect of the Arg426Gln replacement on the catalytic activity of cytochrome P450scc, we reconstituted cholesterol side-chain cleavage activity of the Arg426Gln mutant in the presence of adrenodoxin reductase and adrenodoxin (Fig. 8). In contrast to wild-type cytochrome P450scc, the Arg426Gln mutant is unable to catalyze the cholesterol side-chain cleavage reaction, confirming our data on the inability of this mutant to interact with adrenodoxin and receive electrons, but preserves its ability to interact with cholesterol.

DISCUSSION

The data presented in this paper indicate that the Arg425 and Arg426 residues of cytochrome P450scc, which are located on the proximal surface of the protein near the active site (Fig. 1), are candidate residues for forming the site responsible for electrostatic interaction with adrenodoxin. Thus, site-directed replacement of Arg425 of cytochrome P450scc results in the loss of ability of the cytochrome to correctly interact with the heme group forming the spectrally detected heme protein. The Arg426Gln substitution of cytochrome P450scc leaves intact the spectral and physicochemical properties and the ability to bind cholesterol, but it results in complete loss of the ability to interact with adrenodoxin, receive electrons from reduced ferredoxin, and catalyze the cholesterol side-chain cleavage reaction. This indicates that Arg425, being close to the proximal ligand of cytochrome P450scc (Cys422) (Fig. 9), besides its possible involvement in the interaction with adrenodoxin, appears to play an important structural role in proper coordination of the heme group, since replacement of this residue results in loss by cytochrome P450scc of the ability to bind heme correctly. However, Arg426, located on the surface somewhat further from the active site of cytochrome P450scc (Fig. 9), appears to be directly involved in electrostatic

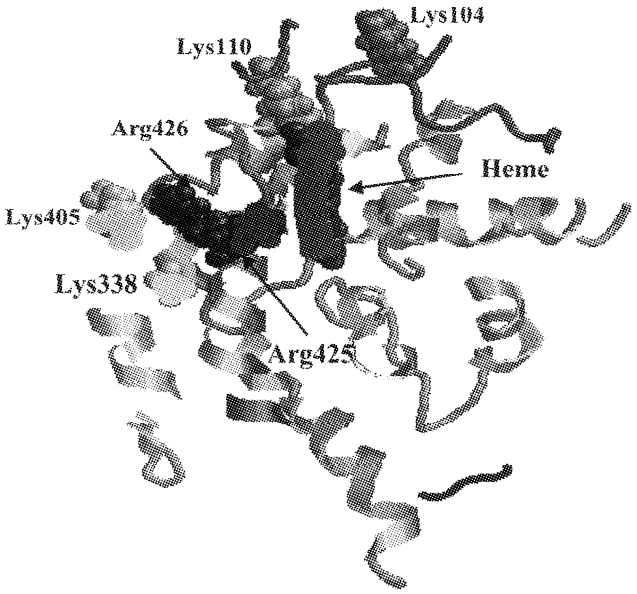


Fig. 9. Location of residues Arg425 and Arg426 in the heme-binding region of cytochrome P450scc from the proximal side based on the model of the 3-dimensional structure of cytochrome P450scc from molecular modeling. Cross-sectional side view.

interaction with a negatively charged group of adrenodoxin, facilitating formation of a specific complex between the two proteins in which an electron is transferred from adrenodoxin to cytochrome P450scc.

The data of the present work support our conclusions made based on analysis of distribution of electrostatic potential on the proximal surface of cytochrome P450scc and indicate the direct involvement of Arg426 in electrostatic interaction of the cytochrome and adrenodoxin, suggesting multiple points of interaction between the two proteins. Figure 10 shows the distribution of the positively charged residues that may be involved in electrostatic interaction with adrenodoxin on the proximal surface of cytochrome P450scc. Residues Arg425 and Arg426, which are located closest to the heme group (Figs. 9 and 10), are identified as a possible sites of interaction in the present work. Additional residues close to the proximal surface of cytochrome P450scc are Lys403 and Lys405; these have been mapped by chemical modification [11] and site-directed mutagenesis [14]. Residues Lys338 and Lys342, which have been mapped by site-directed mutagenesis, are slightly more removed from the heme group but still very important for the interaction with adrenodoxin [13]. The roles of Lys104 and Lys110 are not clear. Chemical modification and site-directed mutagenesis

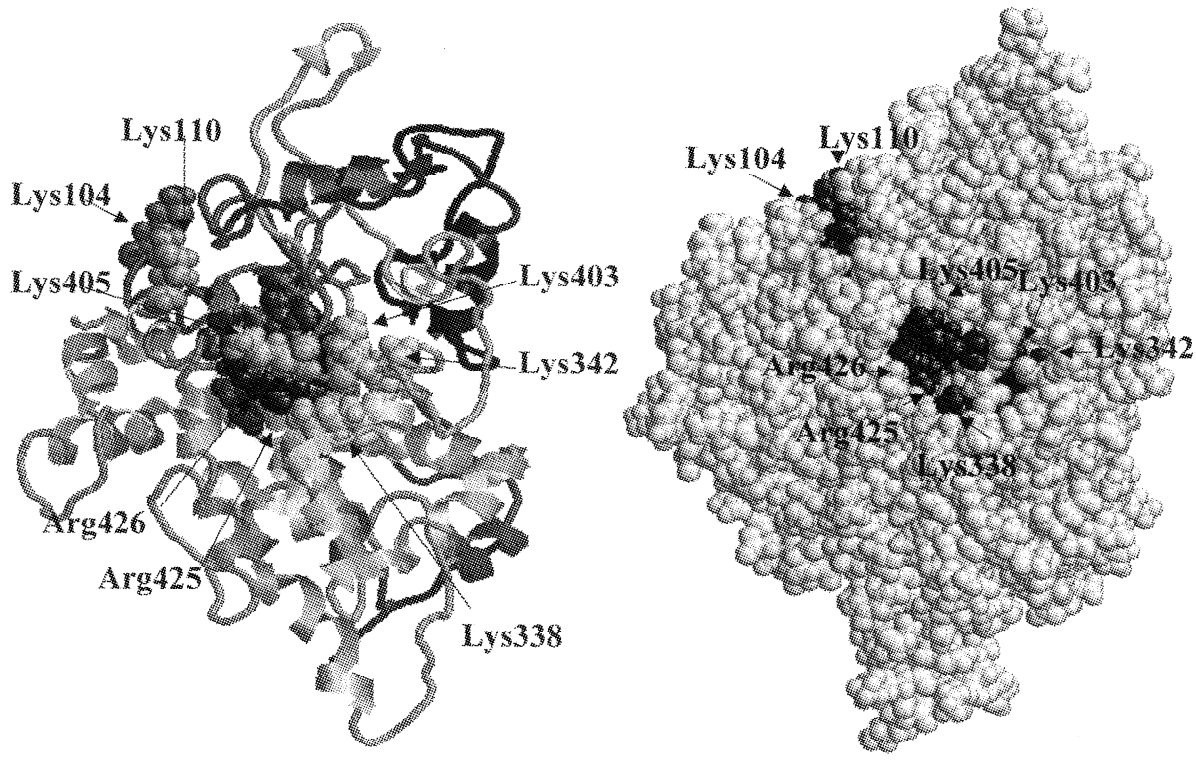
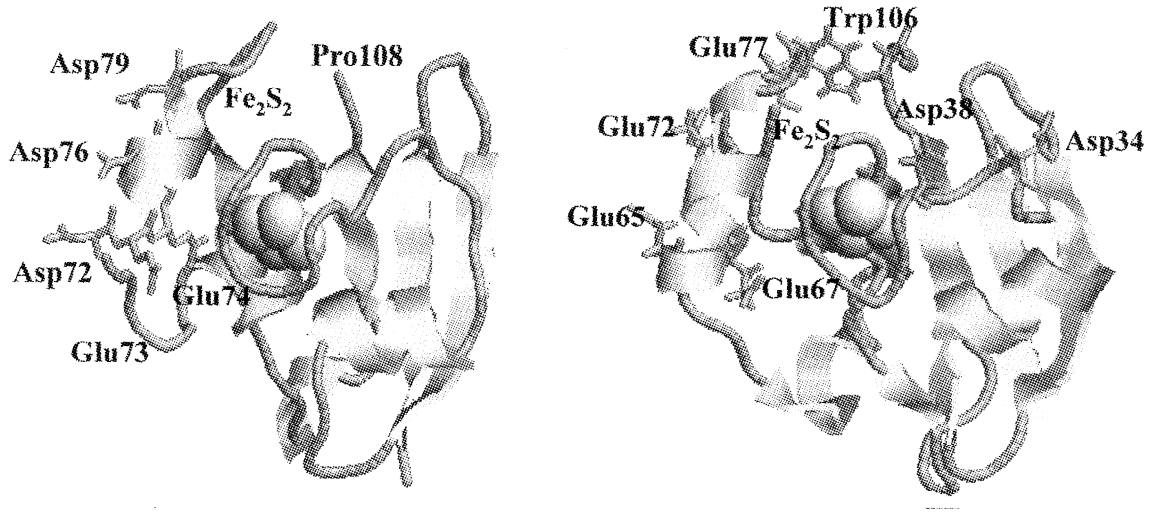


Fig. 10. Distribution of the positively charged residues on the proximal surface of cytochrome P450scc that appear to be involved in the interaction with negatively charged residues of adrenodoxin.

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Adx  9  VHFINRDGETLTTKGKIGDSLLDVVVQNNLDIDGFGACEGTLACSTCHLIFEQHIFEKLE  68
        V +++ DG          G SL+   V N +  D  G C G+ +C+TCH+   +   +K+
Pdx  3  VVYVSHDGTRRELDVADGVSLMQAAVSNGI-YDIVGDCGGSASCATCHVYVNEAFTDKVP  61

Adx 69  AITDEENDMLD-LAYGLTDRSRLGCQICLTKAMDNMTVRVPDAVSDARESIDMGMNSSKIE 128
        A  + E  ML+ +   L   SRL CQI +T  +D + V VPD
Pdx 62  AANEREIGMLECVTAELKPNSRLCCQIIMTPELDGIVVDVPDRQW 106
```

Fig. 11. Alignment of amino acid sequences of bovine adrenodoxin and putidaredoxin from *Ps. putida*. Amino acid sequences of bovine adrenodoxin (ADX1_BOVIN) and putidaredoxin from *Ps. putida* (PUTX_PSEPU) were downloaded from PubMed. The numbering of adrenodoxin is given for its mature form. Comparison of the tertiary structures of adrenodoxin (1AYF) and putidaredoxin (1PUT). Coordinates for adrenodoxin and putidaredoxin were downloaded from Protein Data Bank (PDB). Negatively charged residues of ferredoxin that appear to be participating in electrostatic interactions with cytochrome P450 are indicated.

indicate their possible involvement in the interaction with adrenodoxin, but they are located rather far from the proposed interaction site.

The monooxygenase system of *Pseudomonas putida*, which contains cytochrome P450cam and selectively oxidizes camphor, serves as a model system for studies of the mechanism of electron transfer in mitochondrial monooxygenases of eucaryotes. Thus, it was interesting to compare the mechanisms of interaction of the two different cytochromes P450 with ferredoxins. Putidaredoxin, the bacterial analog of adrenodoxin, is 33-34% homologous to adrenodoxin, contains 106 amino acid residues, and has very similar tertiary structure (Fig. 11). The main part of both ferredoxins is a hydrophobic fragment consisting mostly of β-sheets; a small helical fragment forms the domain responsible for the interaction with the electron transfer partner, the so-called interaction domain.

Earlier studies of the mechanism of interaction of cytochrome P450cam with putidaredoxin have shown that the C-terminal tryptophan residue, Trp106 of putidaredoxin, plays an extremely important role in the interaction of the two proteins [40]. The replacement of Trp106 of putidaredoxin with a non-aromatic residue disturbs the interaction with cytochrome P450cam [41]. However, removal of the C-terminal sequence of adrenodoxin to Pro108 does not affect its interaction with cytochrome P450scc [42]. Site-directed mutagenesis of the negatively charged residues Asp58, Glu65, Glu72, and Glu77 located in the α-helical interaction domain of putidaredoxin, which are homologous to residues Asp79, Asp76, Glu74, Glu73, and Asp72 of adrenodoxin, does not confirm their participation in electrostatic interaction with cytochrome P450cam [43] that was concluded based on chemical modification of putidaredoxin with carbodiimide [44].

Molecular modeling of the complex of cytochrome P450cam with putidaredoxin also indicates that negatively charged residues of the α-helical domain of putidaredoxin homologous to residues of adrenodoxin involved in interaction with cytochrome P450scc do not participate in interaction with cytochrome P450cam. Residues Asp34 and Asp38 of putidaredoxin play an important role in this interaction [45]. The complex of cytochrome P450cam and putidaredoxin is stabilized by three salt bridges—between residues Arg109, Arg112, and Arg79 of cytochrome P450cam and residues Asp38, Asp34, and the C-terminal carboxylic group of Trp106 of putidare-

doxin, respectively. The important role of residues Asp38 and Asp34 of putidaredoxin in the interaction with cytochrome P450cam was confirmed by site-directed mutagenesis [46]. It is interesting that site-directed mutagenesis of residues Lys103,104 and Lys109,110 of cytochrome P450scc, which are located in α-helix C and homologous to residues Arg109 and Arg112 of cytochrome P450cam, does not result in any change in the interaction of cytochrome P450scc with adrenodoxin [14]. Thus, despite significant structural similarities between monooxygenases of pro- and eukaryotes, the mechanism of interaction between electron transfer proteins is strictly individual for each type of monooxygenases and requires the participation of different structural elements of the reacting proteins.

Thus, based on the data presented in this paper and earlier obtained results, it is reasonable to conclude that multi-point electrostatic interactions between negatively charged residues of adrenodoxin and positively charged groups of cytochrome P450scc represented by residues Lys338, Lys442, Lys403, Lys405, and Arg426 play an important role in stabilization of the complex of the two proteins. Now, using molecular docking and the data presented in this paper, we are building a model of the complex of cytochrome P450scc with adrenodoxin.

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REFERENCES

1. Usanov, S. A., Chashchin, V. L., and Akhrem, A. A. (1990) in *Molecular Mechanisms of Adrenal Steroidogenesis and Aspects of Regulation and Application*, Vol. 3 (Ruckpaul, K., and Rein, H., eds.) *Frontiers in Biotransformation*, Akademie-Verlag, Berlin, pp. 1-57.
2. Lambeth, J. D., and Pember, S. (1983) *J. Biol. Chem.*, **258**, 5596-5602.
3. Tuls, J., Geren, L., Lambeth, J. D., and Millet, F. (1987) *J. Biol. Chem.*, **262**, 10020-10025.
4. Tuls, J., Geren, L., Lambeth, J. D., and Millet, F. (1989) *J. Biol. Chem.*, **264**, 16421-16425.
5. Adamovich, T. B., Pikuleva, I. A., Chashchin, V. L., and Usanov, S. A. (1989) *Biokhimiya*, **54**, 1206-1216.
6. Adamovich, T. B., Pikuleva, I. A., Chashchin, V. L., and Usanov, S. A. (1989) *Biochim. Biophys. Acta*, **996**, 247-253.
7. Tsubaki, M., Iwamoto, Y., Hiwatashi, A., and Ichikawa, Y. (1989) *Biochemistry*, **28**, 6899-6907.
8. Millett, F. S., and Geren, L. M. (1991) *Meth. Enzymol.*, **206**, 49-56.
9. Vickery, L. (1997) *Steroids*, **62**, 124-127.
10. Miura, S., Tomita, S., and Ichikawa, Y. (1991) *J. Biol. Chem.*, **266**, 19212-19216.
11. Chashchin, V. L., Turko, I. V., Akhrem, A. A., and Usanov, S. A. (1985) *Biochim. Biophys. Acta*, **828**, 313-324.
12. Guryev, O. L., Dubrovski, T. B., Chernogolov, A. A., Dubrovskaya, S. B., Nicolini, C., and Usanov, S. A. (1997) *Biochemistry (Moscow)*, **62**, 641-647 (Russ.).
13. Wada, A., and Waterman, M. R. (1992) *J. Biol. Chem*, **267**, 22877-22882.
14. Lepesheva, G. I., Azeva, T. N., Strushkevich, N. V., Gilep, A. A., and Usanov, S. A. (2000) *Biochemistry (Moscow)*, **65**, 1409-1418.
15. Geren, L. M., O'Brien, Stonehouerner, J., and Millett, F. (1984) *J. Biol. Chem.*, **259**, 2155-2160.
16. Coghlam, V. M., and Vickery, L. E. (1991) *J. Biol. Chem.*, **266**, 18606-18612.
17. Coghlam, V. M., and Vickery, L. E. (1992) *J. Biol. Chem.*, **267**, 8932-8935.
18. Hasemann, C. A., Kurumbail, R. G., Boduppalli, S. S., Peterson, J. A., and Deisenhofer, J. (1995) *Structure*, **3**, 41-62.
19. Lepesheva, G. I., Azeva, T. N., Strushkevich, N. V., Adamovich, T. B., Cherkesova, T. S., and Usanov, S. A. (1999) *Biochemistry (Moscow)*, **64**, 1079-1088.
20. Lepsheva, G. I., and Usanov, S. A. (1998) *Biochemistry (Moscow)*, **63**, 224-234.
21. Lepesheva, G. I., Strushkevich, N. V., and Usanov, S. A. (1999) *Biochim. Biophys. Acta*, **1434**, 31-43.
22. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
23. Usanov, S. A., Chernogolov, A. A., Lang, M., Passanen, M., Raunio, H., and Pelkonen, O. (1990) *Biokhimiya*, **55**, 865-877.
24. Omura, T., and Sato, R. (1964) *J. Biol. Chem.*, **239**, 2370-2378.
25. Chu, J.-W., and Kimura, T. (1973) *J. Biol. Chem.*, **248**, 2089-2094.
26. Omura, T., and Sato, R. (1967) *Meth. Enzymol.*, **10**, 556-561.
27. Guryev, O., Erokhin, V., Usanov, S., and Nicolini, C. (1996) *Biochem. Mol. Biol. Int.*, **39**, 205-214.
28. Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., and Irace, G. (1984) *Biochemistry*, **23**, 1871-1875.
29. Lepesheva, G. I., and Usanov, S. A. (1996) *Biochemistry (Moscow)*, **61**, 1395-1407 (Russ.).
30. Sugano, S., Morishima, N., Ikeda, H., and Horie, S. (1989) *Anal. Biochem.*, **182**, 327-333.
31. Lepesheva, G. I., and Usanov, S. A. (1997) *Biochemistry (Moscow)*, **62**, 758-768 (Russ.).
32. Peterson, J. A. (1971) *Arch. Biochem. Biophys.*, **144**, 678-693.
33. Muller, A., Muller, J. J., Muller, Y. A., Uhlmann, H., Bernhardt, R., and Heinemann, U. (1998) *Structure*, **6**, 269-280.
34. Pikuleva, I. A., Tesh, K., Waterman, M. R., and Kim, Y. (2000) *Arch. Biochem. Biophys.*, **373**, 44-55.

35. Chernogolov, A. A., and Usanov S. A. (1997) *Biochemistry (Moscow)*, **62**, 1375-1384 (Russ.).
36. Chernogolov, A. A., and Usanov, S. A. (1997) *Biochemistry (Moscow)*, **62**, 1385-1395 (Russ.).
37. Graham, S. E., and Peterson, J. A. (1999) *Arch. Biochem. Biophys.*, **369**, 24-29.
38. Usanov, S. A., Gilep, A. A., Lepesheva, G. I., Graham, S., Peterson, J. A., and Estabrook, R. W. (1999) *Proc. Int. Symp. on Molecular Steroidogenesis*, August 25-28, 1999, Nara, Japan, p. 79.
39. Usanov, S. A., Gilep, A. A., Lepesheva, G. I., Strushkevich, N. V., Graham, S., Peterson, J. A., and Estabrook, R. W. (2000) *Abst. Int. Workshop* "*From Structure to Function: Experimental and Bioinformatic Studies of Cytochrome P450 Superfamily*", July 6-10, 2000, Moscow, Russia, p. 46.
40. Sligar, S. G., Debrunner, P. G., Lipscomb, J. D., Namtvedt, M. J., and Gunsalus, I. C. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 3906-3910.
41. Davies, M. D., and Sligar, S. G. (1992) *Biochemistry*, **31**, 11383-11389.
42. Uhlmann, H., Iametti, S., Vecchio, G., Bonomi, F., and Bernhardt, R. (1997) *Eur. J. Biochem.*, **248**, 897-902.
43. Aoki, M., Ishimori, K., and Morishima, I. (1998) *Biochim. Biophys. Acta*, **1386**, 157-167.
44. Geren, L. M., Tuls, J., O'Brien, Stonehouerner, J., Millett, F., and Peterson, J. A. (1986) *J. Biol. Chem.*, **261**, 15491-15495.
45. Pochapsky, T. C., Lyons, T. A., Kazanis, S., Arakaki, T., and Rasnaswamy, G. (1996) *Biochimie*, **78**, 723-733.
46. Holden, M., Mayhew, M., Bunk, D., Roitberg, A., and Vilker, V. (1997) *J. Biol. Chem.*, **272**, 21720-21725.