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Site-Directed Mutagenesis of Cytochrome P450scc (CYP11A1). Effect of Lysine Residue Substitution on Its Structural and Functional Properties

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Abstract—Our previous chemical modification and cross-linking studies identified some positively charged amino acid residues of cytochrome P450scc that may be important for its interaction with adrenodoxin and for its functional activity. The present study was undertaken to further evaluate the role of these residues in the interaction of cytochrome P450scc with adrenodoxin using site-directed mutagenesis. Six cytochrome P450scc mutants containing replacements of the surface-exposed positively charged residues (Lys103Gln, Lys110Gln, Lys145Gln, Lys394Gln, Lys403Gln, and Lys405Gln) were expressed in *E. coli* cells, purified as a substrate-bound high-spin form, and characterized as compared to the wild-type protein. The replacement of the surface Lys residues does not dramatically change the protein folding or the heme pocket environment as judged from limited proteolysis and spectral studies of the cytochrome P450 mutants. The replacement of Lys in the N-terminal sequence of P450scc does not dramatically affect the activity of the heme protein. However, mutant Lys405Gln revealed rather dramatic loss of cholesterol side-chain cleavage activity, efficiency of enzymatic reduction in a reconstituted system, and apparent dissociation constant for adrenodoxin binding. The present results, together with previous findings, suggest that the changes in functional activity of mutant Lys405Gln may reflect the direct participation of this amino acid residue in the electrostatic interaction of cytochrome P450scc with its physiological partner, adrenodoxin.

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

Cytochrome P450scc (CYP11A1) is the terminal component of the monooxygenase system of the inner mitochondrial membrane; it catalyzes side chain cleavage of cholesterol to form pregnenolone, the precursor of the main steroid hormones, in steroidogenic tissues and gonads [1]. This reaction is a rate-limiting step in the process of steroid hormone biosynthesis and is under the control of a set of regulatory mechanisms. The catalytic cycle of cytochrome P450scc starts from the binding of cholesterol and receipt of the first electron from the physiological partner—the Fe_2S_2 iron-sulfur protein adrenodoxin.

Many studies have been devoted to investigation of the mechanism of interaction of cytochrome P450scc with adrenodoxin [2-12]. Different physicochemical approaches have been used to study the interaction between cytochrome P450scc and adrenodoxin. Chemical modification of surface lysine residues of cytochrome P450scc prevented complex formation between cytochrome P450scc and adrenodoxin [3-7]. Site-directed mutagenesis of Lys^{338} and Lys^{342} (numbering of the "mature" form of cytochrome P450scc) increases the dissociation constant of the cytochrome P450scc–adrenodoxin complex more than 100-fold [13]. On the other hand, chemical modification and site-directed mutagenesis of adrenodoxin suggest the involvement of negatively charged amino acid residues of ferredoxin in electrostatic interaction with cytochrome P450scc [14-16].

Thus, there is much evidence indicating an important role of electrostatic interactions between positively charged amino acid residues of cytochrome P450scc and negatively charged residues of adrenodoxin in the formation of the functional complex between this two proteins, in which electron transfer from adrenodoxin to cytochrome P450scc takes place. Despite the available data, the question of the size of the interacting surfaces and the number of amino acid residues involved in formation of the complex remains unclear.

The aim of the present work was to study using site-directed mutagenesis the functional role of some lysine

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residues in manifestation of the structure and function of cytochrome P450scc. The choice for mutagenesis of the amino acid residues studied in the present work is based on preliminary studies [5, 6, 11], according to which:

a) these amino acid residues are accessible for chemical modification, but become inaccessible in the presence of adrenodoxin;

b) chemical modification of these amino acid residues results in the loss of ability of cytochrome P450scc to bind adrenodoxin.

The data obtained in the present work indicate that substitution of the surface lysine residues of cytochrome P450scc with rare exception does not result in evident disturbances of the tertiary structure of the cytochrome. The data suggest the possible involvement of Lys^{405} of cytochrome P450scc in its interaction 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, glucose-6-phosphate, glucose-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); Bio-Gel HTP (Bio-Rad, USA).

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

K103Q	5′- GTC CTG TTTCAG AAG TCA GGA AC - 3′(sense) GT TCC TGA CTT CTG AAA CAG GAC (anti-sense)
K104Q	5′- GTC CTG TTT AAG CAG TCA GGA ACC TGG -3′ CCA GGT TCC TGA CTG CTT AAA CAG GAC
K109Q	5′- CA GGA ACC TGG CAG AAA GAC CGG GTG G -3′ C CAC CCG GTC TTT CTG CCA GGT TCC TG
K110Q	5′- GGA ACC TGG AAG CAA GAC CGG GTG G -3′ C CAC CCG GTC TTG CTT CCA GGT TCC
K145Q	5′- GC CTC CTG CAC CAG CGC ATC AAG C -3′ G CTT GAT GCG CTG GTG CAG GAG GC
K148Q	5′- G CAC AAG CGC ATC CAG CAG CAG GGC -3′ GCC CTG CTG CTG GAT GCG CTT GTG C
K372Q	5′- C CTG ATT CCT GCC CAG ACA CTG GTG C -3′ G CAC CAG TGT CTG GGC AGG AAT CAG G
K394Q	5′- CC AGT CCG GAC CAG TTT GAC CCA ACC -3′ GT TGG GTC AAA CTG GTC CGG ACT GG
K403Q	5′- GG TGG CTG AGT CAA GAC AAA GAC CTC -3′ GAG GTC TTT GTC TTG ACT CAG CCA CC
K405Q	5′- GG CTG AGT AAA GAC CAA GAC CTC ATC C -3′ G GAT GAG GTC TTG GTC TTT ACT CAG CC

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

Expression and purification of proteins. Recombinant proteins of the cytochrome P450-dependent monooxygenase system from bovine adrenocortical mitochondria—cytochrome P450scc, adrenodoxin, and adrenodoxin reductase—have been 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 plasmids containing cDNA encoding mature adrenodoxin (pBa1159) and adrenodoxin reductase (pBAR1607) were kindly provided by Prof. A. Sagara (Kochi Medical School, Japan).

During expression of adrenodoxin reductase in *E. coli*, the cells after induction with 0.2 mM IPTG were incubated for 48 h at 28°C with stirring at 120 rpm. The cells were harvested by centrifugation and suspended in buffer A (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.5 mM PMSF) at cell/buffer ratio 1 : 6. Sonicated cells were supplemented with $K_3Fe(CN)_6$ at the final concentration 5 mM to oxidize adrenodoxin reductase, which is expressed in *E. coli* in the reduced state, and the suspension was dialyzed against buffer A. The remainder of the membrane was removed by centrifugation, and the supernatant was applied to adrenodoxin-Sepharose. Adrenodoxin reductase was eluted from adrenodoxin-Sepharose using buffer A containing 0.6 M NaCl and 0.3% sodium cholate. Fractions containing adrenodoxin reductase were collected, diluted 10-15 times with 10 mM phosphate buffer, pH 7.4 (buffer B), and applied to a column with 2′,5′-ADP-Sepharose 4B. The column was washed with buffer B containing 0.05 M NaCl. Adrenodoxin reductase was eluted from the 2′,5′-ADP-Sepharose 4B with buffer B containing 0.5 M NaCl. The resulting highly purified adrenodoxin reductase was characterized by spectrophotometric index (A_{271}/A_{450}) of 7.2.

Adrenodoxin was purified from *E. coli* cells as previously described [17]. Highly purified adrenodoxin was characterized by spectrophotometric index A_{414}/A_{280} of 0.94.

Isolation and purification of cytochrome P450scc (wild-type and mutant forms) was carried out according to a scheme developed for purification of the substrate-bound high-spin form of the heme protein [18, 19] including sequential ultrasonic treatment of *E. coli* cells, solubilization of sonicated lysate with Emulgen 913, precipitation of solubilized the cytochrome P450scc with polyethylene glycol (6 kD) at final concentration 20%, incubation of the solubilized pellet with cholesterol, and affinity chromatography on adrenodoxin-Sepharose 4B.

Analytical methods. The protein content of bacterial cells after expression was determined and the purity of final preparations was monitored using SDS PAGE in

10% gel according to Laemmli [20] using a Protean II apparatus (Bio-Rad). Immunochemical identification of recombinant proteins was done by immunoblotting [21]. The concentration of cytochrome P450scc, adrenodoxin reductase, and adrenodoxin was 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 [22, 23].

Spectral characterization of cytochrome P450scc mutants. Spectrophotometric measurements were made using a Shimadzu UV-3000 spectrophotometer (Shimadzu, Japan). The concentration of cytochrome P450scc and its denatured form—cytochrome P420—was determined from the carbon monoxide difference spectra of the dithionite-reduced heme protein 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 [24]. The recording of reduced carbon monoxide difference spectra was also used to assess the expression level of cytochrome P450scc in *E. coli* cells as well as to determine cytochrome P450scc concentration at the intermediate steps of its isolation and purification. The content of the high-spin form of cytochrome P450scc in the purified cytochrome P450scc preparation was calculated from absolute absorbance spectra using the ratio $\Delta A_{(390\text{-}470)}/\Delta A_{(416\text{-}470)}$ [25] equal 0.4 and 2.2 for the low- and high-spin forms of cytochrome P450scc, respectively. The ratio of peak intensities tyrosine/tryptophan $\Delta A_{(287\text{-}283)}/\Delta A_{(295\text{-}290.5)}$ in the second derivatives of the ultraviolet spectra of cytochrome P450scc was determined according to [26].

The circular dichroism spectra were recorded using a JASCO J-720 spectropolarimeter (JASCO, Japan) under the following conditions: slit, 1 nm; time of response, 2 sec; scanning rate, 20 nm/min; temperature, 20°C. In the ultraviolet region (195-250 nm), the spectra were recorded using a 1-mm optical path and concentration of cytochrome P450scc 1 μM in 5 mM potassium phosphate buffer, pH 7.2. For recording of spectra in the visible region (300-600 nm), cells with optical path 10 mm were used, and the concentration of cytochrome P450scc was 10 μM in 10 mM potassium phosphate buffer, pH 7.2. The molar ellipticity [Θ] was calculated as:

$$[\Theta] = \Theta/(10\cdot C\cdot l),$$

where Θ is ellipticity (millidegree), C is protein concentration (M), and l is the optical path length (cm).

The contents of the elements of secondary structure in cytochrome P450scc were calculated from the ultraviolet circular dichroism spectra using the program Dichropro 2.5.

Limited proteolysis of cytochrome P450scc and its mutant forms with trypsin. Limited proteolysis of cytochrome P450scc with trypsin was carried out in 50 mM potassium phosphate buffer, pH 7.4, at final cytochrome P450scc concentration 10 μM at 18°C and trypsin/cytochrome P450scc molar ratio 1 : 50. Trypsin concentration in solution was determined spectrophotometrically using molar extinction coefficient $\varepsilon_{280} = 3.5\cdot 10^4$ $M^{-1}\cdot cm^{-1}$. Trypsinolysis was stopped by adding a 4-fold excess of soybean trypsin inhibitor. The products of proteolytic modification of cytochrome P450scc were analyzed using SDS PAGE in 12% gel, staining with Coomassie Blue R-250, and scanning at 550 nm.

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 and chemically reduced cytochrome P450scc [27].

Determination of cholesterol side chain cleavage activity of cytochrome P450scc and its mutant. Cholesterol side chain cleavage activity of cytochrome P450scc and its mutant forms was analyzed according to [28] by determining using HPLC on a Zorbax-Sil column (4.6 × 250 mm) in the system hexane–isopropanol (3 : 1) the amount of progesterone formed from the reaction product, pregnenolone, in the presence of cholesterol oxidase. Deoxycorticosterone was used as internal standard.

Registration of the spin changes in cytochrome P450scc during interaction with adrenodoxin. Spectral changes in cytochrome P450scc induced by adrenodoxin were recorded as previously described [29]. The apparent dissociation constants for cytochrome P450–adrenodoxin complex were determined in 20 mM HEPES, 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 $\Delta A_{100} - \Delta\varepsilon_{(390\text{-}420\ nm)} = 110$ $mM^{-1}\cdot cm^{-1}$ was accepted as a 100% change of the spin state [30]. The absence of denatured cytochrome P450scc in the sample was proved by recording of the reduced carbon monoxide difference spectrum.

The changes in the free energy of binding of cytochrome P450scc and its mutant forms with adrenodoxin were calculated based on the equation [9]:

$$\Delta(\Delta G) = -RT\ln(K_d^{\text{wild-type}}/K_d^{\text{mutant}}),$$

where $R = 1.98\cdot 10^{-3}$ $kcal\cdot mole^{-1}\cdot deg^{-1}$, and $T = 298K$ (25°C).

RESULTS AND DISCUSSION

Experiments using chemical modification and cross-linking as well as site-directed mutagenesis have demonstrated the participation of the negatively charged amino

acid residues of adrenodoxin in its interaction with cytochrome P450scc; the sensitivity of complex formation to ionic strength indicates an important role of electrostatic interactions in the binding of the ferredoxin and the heme protein. The presence of a cluster of negatively charged amino acid residues in the adrenodoxin molecule, which includes residues Asp^{79}, Asp^{76}, Glu^{74}, Glu^{73}, and Asp^{72}, clearly indicates the involvement of positively charged amino acid residues of cytochrome P450scc in its interaction with the ferredoxin. Elucidation of the crystal structure of the truncated [31] and full-length forms [32] of adrenodoxin supports the presence in the structure of adrenodoxin of negatively charged α-helix that includes negatively charged amino acid residues Asp^{79}, Asp^{76}, Asp^{72}, Glu^{74}, and Glu^{73}, previously identified as residues that appear to be responsible for interaction of adrenodoxin with cytochrome P450scc and adrenodoxin reductase [9, 14-16].

Our previous studies of the interaction between cytochrome P450scc and adrenodoxin using chemical modification and chemical cross-linking identified some positively charged amino acid residues of cytochrome P450scc [6, 11] that might be involved in its interaction with adrenodoxin. These amino acid residues are randomly distributed in the structure of cytochrome P450scc, and during alignment of structures of cytochrome P450scc with structures of cytochrome P450 with known crystal structures the following structural elements of cytochrome P450 are located: Lys^{103} and Lys^{104} (α-helix B′), Lys^{109} and Lys^{110} (α-helix C), Lys^{145} and Lys^{148} (α-helix D), Lys^{394} (α-helix K′). Residues Lys^{403} and Lys^{405} are located in a structural element called the meander [33, 34], which is located on the proximal surface of cytochrome P450 near the heme-binding sequence and, thus, is perhaps involved in interaction with electron-transfer partners [34]. Figures 1 and 2 show the alignments of the amino acid sequence of cytochrome P450scc with amino acid sequences of the cytochromes P450 of mitochondrial type in the N- (Fig. 1) and C-terminal sequence (Fig. 2). As follows from these alignments, the lysine residues, which have been mapped by us, are rather conservative in the structure of different cytochromes P450 of mitochondrial type, suggesting that these residues are functionally important.

To further understand the functional relevance of these amino acid residues of cytochrome P450scc, we used site-directed modification of these residues. Figure 3 shows the data on expression level of cytochrome P450scc and its mutant forms in *E. coli* cells (a) and SDS PAGE of the proteins of recombinant cells (b). The expression level for mutants of cytochrome P450scc is very similar to that of for the wild-type protein, indicating the absence of the major changes in the conformation of cytochrome P450scc after replacement of the lysine residues and retention of the native structure by the modified proteins. However, substitution of Lys^{109} to Gln is followed by the absence of spectrally detected cytochrome P450scc in *E. coli* cells, while the presence of apo-cytochrome P450scc in these cells is proved by SDS PAGE and immunoblotting analysis (data not shown). Thus, substitution of Lys^{109} results in dramatic changes in the folding and heme insertion in the molecule of this cytochrome P450scc mutant. This is a very interesting phenomenon since

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sccbov     85  PPWLAYHRYYQKPIGVLFKKSGTWKKDRVVLNTEVMAPEAIK 126
sccsheep  124  PPWLAYHRYYQKPIGVLFKKSGAWKKDRVVLNTEVMAPEAIK 165
sccgoat   124  PPWLAYHQYYQKPIGVLFKKSGAWKKDRVVLNTEVMAPEAIK 165
sccpig    124  PPWVAYHQHYQKPVGVLLKKSGAWKKDRLVLNTEVMAPEAIK 165
scchum    124  PPWVAYHQYYQRPIGVLLKKSAAWKKDRVALNQEVMAPEATK 165
scchorse  124  PPWTAYHQYFQKPVGVLFKKSSDAWKKDRLALNPEVMALESIK 165
11B1hum   115   PWVAYRQHRGHKCGVFLLNGPEWRFNRLRLNPEVLSPNAVQ 155
11B1bov   115   PWLAYRQARGHKCGVFLLNGPQWRLDRLRLNPDVLSLPALQ 155
11B2hum   115   PWVAYRQHRGHKCGVFLLNGPEWRFNRLRLNPDVLSPKAVQ 155
11B2rat   125   PWVAHRELRGLRRGVFLLNGAEWRFNRLKLNPNVLSPKAVQ 165
24hum     132   PWKAYRDYRKEGYGLLILEGEDWQRVRSAFQKKLMKP     168
24chick   123   PWKAYRDYRDEGYGLLILEGKDWQRVRSAFQKKLMKPKEV  162
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Fig. 1. Alignment of amino acid sequences of mitochondrial cytochromes P450 in the N-terminal sequence including amino acid residues of cytochrome P450scc that have been subjected to site-directed mutagenesis in the present work: Lys^{103}, Lys^{104}, Lys^{109}, and Lys^{110}. The amino acid sequences of mitochondrial cytochromes P450 were downloaded from Medline. Numbering for cytochrome P450scc is according to the "mature" form of the heme protein, while the other cytochromes P450 are presented as precursors.

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sccbov    387 AFFSSPDKFDPTRWLSKDKD-----LIHFRNLGFGWGVRQCV 423
sccgoat   426 AFFSNPDKFDPTRWLGKDKD-----LIHFRNLGFGWGVRQCV 462
sccsheep  426 AFFSNPDKFDPTRWLGKDKD-----LIHFRNLGFGWGVRQCV 462
sccpig    426 AFFSNPGQFDPTRWLGKERD-----LIHFRNLGFGWGVRQCV 462
scchorse  427  FFSNPRRFDPTRWLDKNKD-----LTHFRNLGFGWGVRQCL 462
scchum    428  FFFDPENFDPTRWLSKDKN-----ITYFRNLGFGWGVRQCL 463
11B1bov   415 AVFARPESYHPQRWLDRQGS-----GSRFPHLAFGFGVRQCL 451
11B2rat   425 AVFPRPERYMPQRWLERKRS--------FQHLAFGFGVRQCL 458
24mouse   429   FEDADKFRPERWLEKEKK-----INPFAHLPFGVGKRMCI 463
24rat     429   FEDSHKFRPERWLQKEKK-----INPFAHLPFGIGKRMCI 463
25hum     437   FSEPESFQPHRWLRNSQPATPRIQHPFGSVPFGYGVRACL 476
27rat     448   FPEPESFQPHRWLRKREDDNSGIQHPFGSVPFGYGVRSCL 487
27hum     438   FSEPESFQPHRWLRNSQPATPRIQHPFGSVPFGYGVRACL 477
1hum      420 AQFPEPNSFRPARWLGEGPT-----PHPFASLPFGFGKRSCM 456
1rat      413 AQFREPNSFNPARWLGEGPA-----PHPFASLPFGFGKRSCI 449
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Fig. 2. Alignment of amino acid sequences of mitochondrial cytochromes P450 in the C-terminal sequence including amino acid residues of cytochrome P450scc that have been subjected to site-directed mutagenesis in the present work, P450scc Lys^{394}, Lys^{403}, and Lys^{405}. Amino acid sequences of mitochondrial cytochromes P450 were downloaded from Medline. Numbering for cytochrome P450scc is according to the "mature" form of the heme protein, while the other cytochromes P450 are presented as precursors.

mutagenesis of the next lysine residue of cytochrome P450scc, Lys^{110}, has practically no affect on the folding and heme insertion into that mutant of cytochrome P450scc, and this mutant form of cytochrome P450scc is normally expressed in *E. coli* cells. Replacement of Lys^{148} for Gln in cytochrome P450scc does not affect expression level of the cytochrome in *E. coli* cells, and cytochrome P450scc is spectrally seen in the cells by recording the

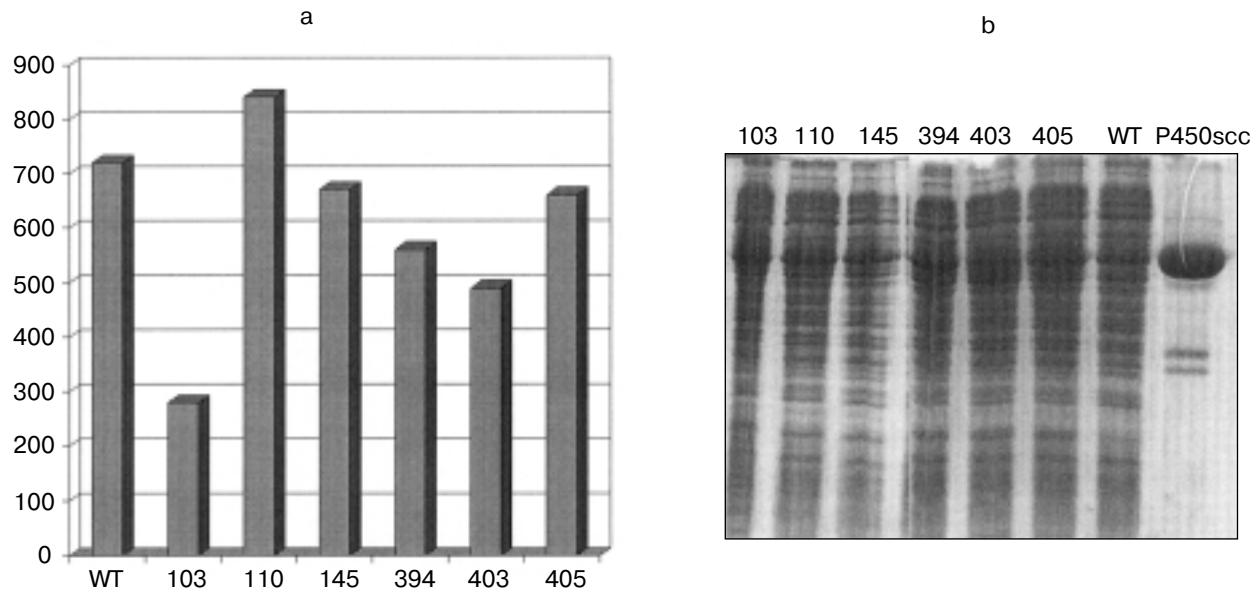

Fig. 3. Spectrophotometrically detected expression level (nmole per liter of culture) of the mutant forms of cytochrome P450scc in *E. coli* cells (a) and SDS PAGE of total proteins in a 12% gel (b).

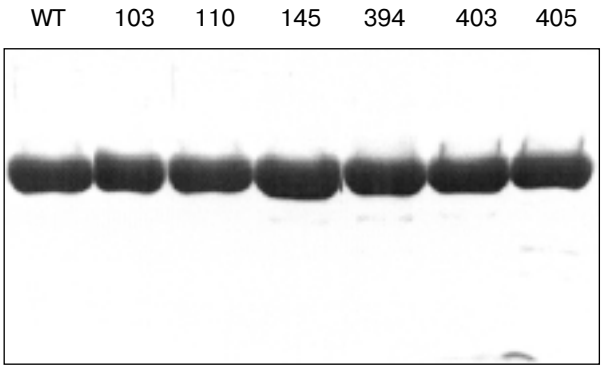

Fig. 4. SDS PAGE of purified mutant forms of cytochrome P450scc in 10% gel.

reduced carbon monoxide difference spectra, but this form of cytochrome P450scc is characterized by extreme instability and it is denatured very rapidly, making purification of this cytochrome P450scc mutant very problematic. Replacement of Lys^{104} for Gln in cytochrome P450scc results in a dramatic decrease in the expression level of this cytochrome mutant in *E. coli* cells (120 nmoles of cytochrome P450scc per liter of culture). For this reason, this mutant of cytochrome P450scc was not used for purification.

The highly purified mutant forms of cytochrome P450scc are in native form and contain practically no denatured protein as follows from the reduced carbon monoxide difference spectrum. The only exception was the Lys103Gln cytochrome P450scc mutant, the content of cytochrome P420 in its preparation being about 22%. This indicates that replacement of Lys103 decreases the stability of cytochrome P450scc even in the presence of cholesterol. The spectral properties of highly purified mutants of cytochrome P450scc, the homogeneity of which is proved by SDS PAGE (Fig. 4), are summarized in Table 1. The absolute absorbance spectra of mutants of cytochrome P450scc are presented in Fig. 5. As follows from Table 1, all the cytochrome P450scc mutants are mainly in the high-spin state, which is explained by the use of cholesterol in the buffer during purification, although the content of the high-spin form varied from 59% in mutant Lys103Gln to 95% in mutant Lys394Gln and 98% in the wild-type cytochrome P450scc. The absence of significant differences in the second derivative of the ultraviolet spectrum, which is very sensitive to any changes in protein conformation [26] and reflects the average polarity of the microenvironment of the tyrosine residues present in cytochrome P450scc, shows the absence of major changes in the conformation of the mutant forms of cytochrome P450scc.

To confirm the absence of dramatic conformational changes in the cytochrome P450scc mutants, we also used limited proteolysis with trypsin and circular dichroism spectroscopy. It was shown earlier that changes in the three-dimensional structure of cytochrome P450scc result in changes in the rate of limited proteolysis of cytochrome P450scc by trypsin [18]. Thus, the time necessary for 50% cleavage of substrate-bound recombinant cytochrome P450scc was four times greater than the time for 50% cleavage of substrate-free cytochrome P450scc. Based on these data, we suggested that any changes in the three-dimensional structure which might result from the site-directed mutagenesis of cytochrome P450scc should be necessarily reflected in the accessibility of the cytochrome P450scc mutant to trypsin. However, as follows from experiments on limited proteolysis of cytochrome P450scc and its mutant forms, both the wild-type cytochrome P450scc and all mutants have the same limited proteolysis pattern, indicating the absence of dramatic conformational changes in cytochrome P450scc after its modification (Fig. 6).

One of the most informative methods for determination of changes in the secondary [35, 36] and tertiary [37,

Table 1. Spectral properties of cytochrome P450scc mutants

P450scc	$i_{393/280}$	High spin content, %	Second derivative: $(A_{287\text{-}283})/(A_{295\text{-}290.5})$	C_{CO}/C_{abs}, %
Wild type	0.86	98	1.26	62
Lys103Gln	0.80	58	1.13	66
Lys110Gln	0.84	73	1.2	68
Lys145Gln	0.81	68	1.18	74
Lys394Gln	0.87	95	1.18	60
Lys403Gln	0.79	86	1.12	65
Lys405Gln	0.69	84	1.01	67

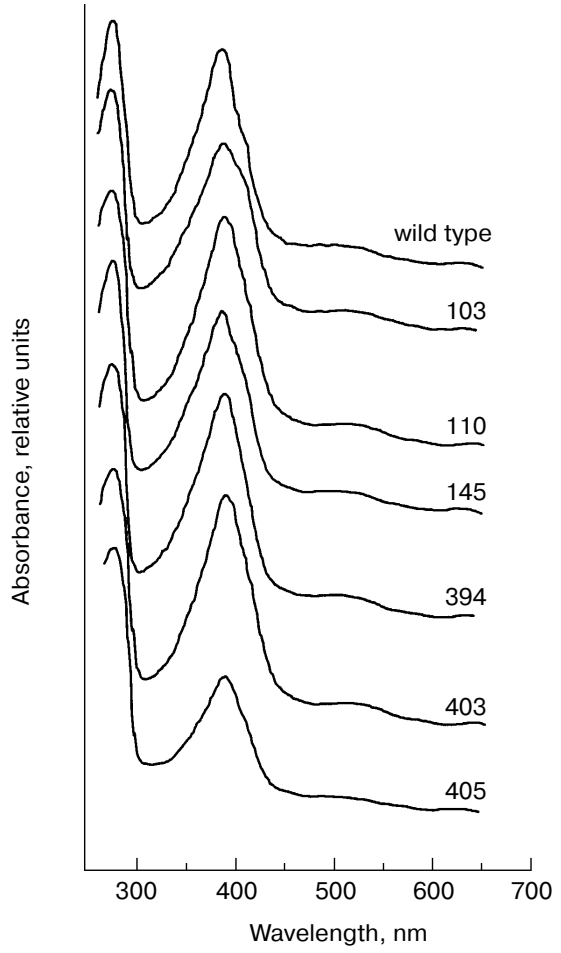

Fig. 5. Absolute absorbance spectra of purified cytochrome P450scc mutants in 50 mM potassium phosphate buffer, pH 7.4. Protein concentration, 2 μM.

38] structure of proteins is circular dichroism. Circular dichroism was used to study changes in the microenvironment of the heme pocket of cytochrome P450 as affected by temperature [39], substrate binding [40], and the transition of heme iron from the oxidized to reduced state [41]. It was shown that the increase in the negative ellipticity in the Soret region (380-500 nm) reflects the approach of the neighbor aromatic amino acid residues to the heme [41, 42], while the increase in the ratio of the intensity of the two bands at 410 and 350 nm (Soret/delta) appear to be the result of formation an additional hydrogen bond between the sixth, axial ligand of the heme and I-helix [43].

The circular dichroism spectra of the cytochrome P450scc mutants are presented in Fig. 7. In the ultraviolet region (190-250 nm), all spectra were practically identical, indicating the absence of dramatic changes in the conformation of cytochrome P450scc after site-directed mutagenesis. Some differences in the circular dichroism spectra in the visible region (300-600 nm) seem to reflect differences in the spin state of the purified cytochrome P450scc mutants (Table 1).

The absence of evident changes in the ultraviolet circular dichroism spectra confirms the conclusion that site-directed mutagenesis of the surface lysine residues of cytochrome P450scc does not results in dramatic changes in the secondary structure of cytochrome P450scc. The calculation of the elements of secondary structure of cytochrome P450scc from the circular dichroism spectra according to the method of Bolotina gave the following results: α-helix, 62%; β-sheet, 7%; β-turn, 19%, and coil, 15%. The similarity of the visible circular dichroism spectra of cytochrome P450scc and its mutants confirms our suggestion that the microenvironment of the heme pocket is not dramatically changed after the mutagenesis. Similar rates of proteolytic modification of cytochrome P450scc and its mutants also confirm the similarity of the three-dimensional structure of all these heme proteins as well as similar accessibility of the polypeptide chain Arg^{250}-Arg^{257} to trypsin.

Thus, the data indicate that site-directed mutagenesis of the surface lysine residues located predominantly in the N-terminal sequence of cytochrome P450scc does not result in any evident changes of the folding of the heme protein and the content of its elements of secondary structure. This allows us to ascribe the changes in the interaction of cytochrome P450scc with adrenodoxin after site-directed mutagenesis of the cytochrome only to the changes in complex formation between the heme protein and the ferredoxin.

The functional properties of cytochrome P450scc mutants are summarized in Table 2. For cytochrome

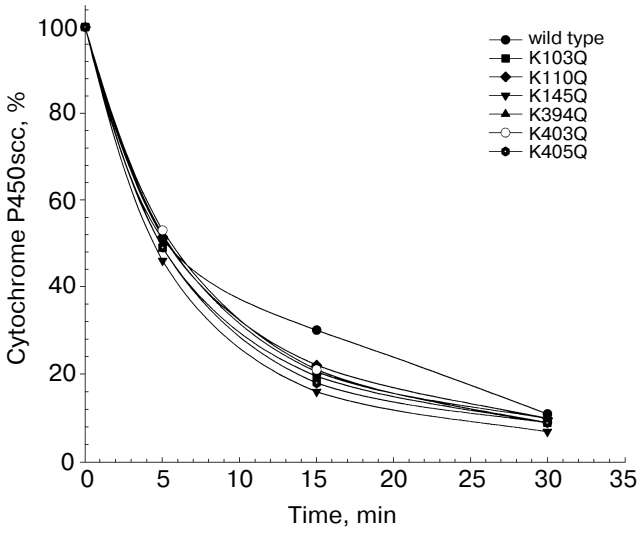

Fig. 6. Limited proteolysis of cytochrome P450scc mutants (10 μM) with trypsin in 50 mM potassium phosphate buffer, pH 7.4, at 18°C and trypsin/cytochrome P450scc ratio 1 : 50 (mole/mole).

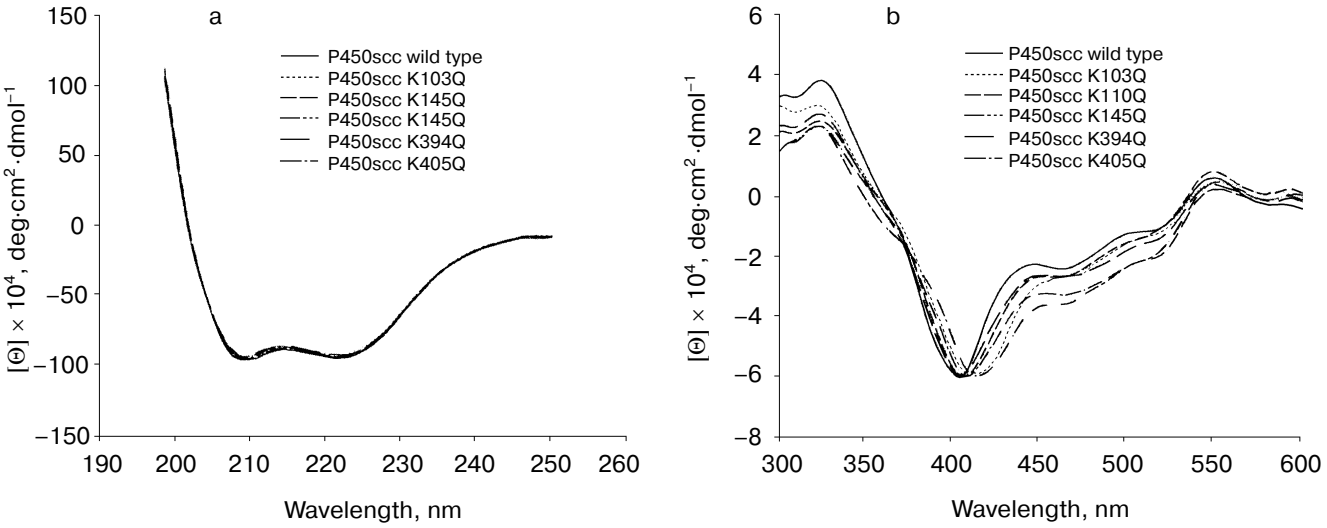

Fig. 7. Circular dichroism spectra of cytochrome P450scc mutants in (a) ultraviolet and (b) visible region. Concentration of cytochrome P450scc is 5 and 10 μM, respectively; the optical path length is 1 and 10 mm, respectively.

P450scc mutants Lys103Gln, Lys110Gln, Lys145Gln, Lys394Gln, and Lys403Gln, changes in functional properties as compared to the wild-type protein are not evident: the cholesterol side-chain cleavage activity varies between 86% for the Lys145Gln mutant and 69% for the Lys403Gln mutant; the changes in the efficiency of enzymatic reduction of cytochrome P450scc in the reconstituted system containing NADPH-adrenodoxin reductase and adrenodoxin, reflecting the effect of amino acid substitution on the first electron transfer, do not exceed 30%. The interaction of the cytochrome P450scc mutants with adrenodoxin-Sepharose 4B, reflecting the physical interaction of the cytochrome P450scc mutants to immobilized adrenodoxin, consists of 70-80% compared to the wild-type protein. More variable are the dissociation constants for the complex between adrenodoxin and cytochrome P450scc mutants as determined by spectrophotometric titration.

Of significant interest are the results on the absence of major changes in the interaction of cytochrome P450scc with adrenodoxin after modification of Lys^{110}. Comparison of the structures of cytochromes P450scc and P450cam indicates that Lys^{110} of cytochrome P450scc coincides with Arg^{112} of cytochrome P450cam, which has been shown by site-directed mutagenesis and molecular modeling to directly participates in electrostatic interaction with Asp^{38} of putidaredoxin [44-46]. Does this indicate that the mechanism of interaction of putidaredoxin

Table 2. Functional properties of cytochrome P450scc mutants

Cytochrome P450scc	Interaction with adrenodoxin-Sepharose, %	Enzymatic reduction in reconstituted system, %	Dissociation constant for adrenodoxin, %	Cholesterol side chain cleavage activity, %
Wild type	80	100	100	100
Lys103Gln	85	95	64	71
Lys110Gln	78	105	120	69
Lys145Gln	66	87	100	86
Lys394Gln	75	80	49	71
Lys403Gln	58	69	67	69
Lys405Gln	35	25	14	30

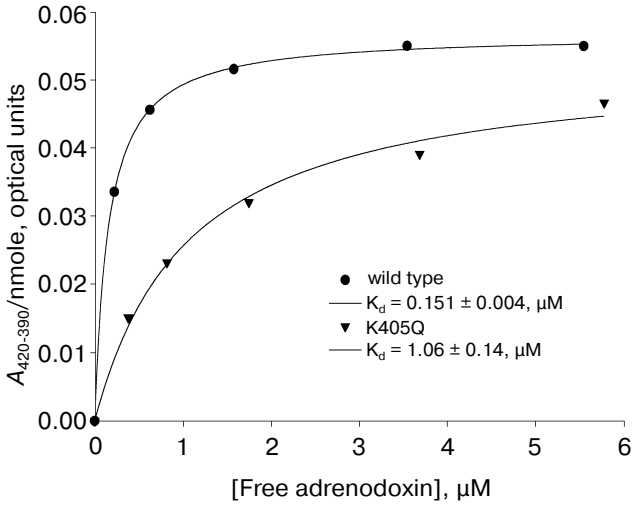

Fig. 8. Titration curves for interaction of wild-type cytochrome P450scc and Lys405Gln cytochrome P450scc mutant with adrenodoxin. Titration of cytochrome P450scc and Lys405Gln cytochrome P450scc mutant with adrenodoxin was performed in 20 mM HEPES, pH 7.2, containing 0.1% Tween-20, 0.1 mM EDTA, 0.1 mM DTT, 50 mM sodium chloride, and 20 μM cholesterol. The cytochrome P450scc concentration was 1 μM.

and adrenodoxin with cytochromes P450cam and P450scc, respectively, is different? It is known that putidaredoxin is not able to substitute for adrenodoxin in the cholesterol side chain cleavage reconstitution system.

At the same time, substitution of Lys^{405} for Gln in cytochrome P450scc results in a 3.3-fold decrease in cholesterol side chain cleavage activity and 4-fold decrease in efficiency of enzymatic reduction of the cytochrome P450scc mutant with adrenodoxin. The interaction of the Lys405Gln cytochrome P450scc mutant with immobilized adrenodoxin as compared to the wild-type protein is only 35%.

The spectrophotometric titration curves obtained on the binding of the wild-type cytochrome P450scc and the Lys405Gln cytochrome P450scc mutant with adrenodoxin are presented in Fig. 8. The dissociation constants for the complex between the wild-type cytochrome P450scc and adrenodoxin (0.151 ± 0.004 μM) and the Lys405Gln cytochrome P450scc mutant and adrenodoxin (1.06 ± 0.14 μM) calculated from these curves differ more that 7-fold. The neutralization of lysine residue Lys^{405} in cytochrome P450scc results in an increase in the free energy of the binding with adrenodoxin (ΔG) by 1.15 kcal·$mole^{-1}$, confirming the disturbance of electrostatic interactions between cytochrome P450scc and adrenodoxin after replacement of Lys^{405}. The value of the change in the free energy of binding for the Lys^{405} cytochrome P450scc mutant and adrenodoxin (ΔG = 1.15 kcal·$mole^{-1}$) is in good agreement with the changes of this parameter after neutralization of the "acidic" residues Asp^{76} and Asp^{79} of adrenodoxin in the interaction of ferredoxin mutants with cytochrome P450scc [9]. This fact is indirectly confirms the disturbance of electrostatic interactions between cytochrome P450scc and adrenodoxin resulting from replacement of Lys^{405}.

It should be noted that the functional parameters of other cytochrome P450scc mutants (Lys394Gln and Lys403Gln) containing substitutions close to Lys^{405} are also decreased compared to the wild-type cytochrome P450scc, but to much less extent. Moreover, a recently published paper indicates that an arginine residue (Arg^{418}) of cytochrome P450c27, another representative of the group of mitochondrial cytochromes P450, which participates in 27-hydroxylation of cholesterol, is extremely important for electrostatic interaction of this type of cytochrome P450 with adrenodoxin [47]. The alignment of the structures of cytochromes P450scc and P450c27 in this region (Fig. 2) indicates that arginine residue Arg^{418} in cytochrome P450c27 is located in the same region as Lys^{403} and Lys^{405} in cytochrome P450scc, confirming the importance of these residues of mitochondrial cytochromes P450 for electrostatic interaction with negatively charged residues of ferredoxin.

Thus, the data presented in the present work indicate an important role of lysine residues Lys^{403} and Lys^{405} in the electrostatic interactions of cytochrome P450scc with adrenodoxin and, keeping in mind the importance of some other positively charged residues of cytochrome P450scc in the interaction with adrenodoxin (Lys^{338} and Lys^{342}), indicate the multi-point character of interaction of these two proteins.

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