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ARTICLES

Site-Directed Mutations (Asp405Ile and Glu124Ile) in Cytochrome P450scc: Effect on Adrenodoxin Binding

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Abstract Cytochrome P450scc, mitochondrial adrenodoxin (Adx), and adrenodoxin reductase (AdR) are an essential components in a steroid hydroxylation system. In particular, mytochondrial cytochrome P450scc enzyme catalyses the first step in steroid hormones biosynthesis, represented by the conversion of cholesterol to pregnenolone. In order to study the effect of single mutations on the Adx binding a model of bovine cytochrome P450scc, previously optimized by molecular modeling, was utilized. It was hypothesized by molecular docking that two residues (Asp405 and Glu124) are involved in Adx binding. By site-directed mutagenesis, two mutants of cytochrome P450scc (Asp405Ile and Glu124Ile) expressed in *Escherichia coli*, were realized by replacing with isoleucines. The site-directed mutations effect on Adx binding was evaluated by differential spectral titration. The apparent dissociation constant values for Asp405Ile and Glu124Ile cytochrome P450scc show that the mutated residues seem to be at the interaction domain with Adx or at least close to it, as predicted by molecular modeling study. Finally, the engineered enzymes were characterized by biochemical and biophysical techniques such as circular dichroism (CD), UV/Vis spectroscopy, and electrochemical analysis. J. Cell. Biochem. 95: 720–730, 2005.

Key words: cytochrome P450scc; molecular modeling; site-directed mutagenesis; UV-Vis spectroscopy

Cytochrome P450 monooxygenases enzymes (P450s) play a pivotal role in the biosynthesis of steroids, fatty acids, prostaglandins, and secondary metabolites of plants and microorganisms, as well as in the detoxification of a wide range of xenobiotic compounds as drugs or chemical pollutants [Strobel et al., 1995]. Cytochrome P45011A1, member of CYP11 family, is commonly namely P450scc or cholesterol side chain cleavage enzyme or CYP11A1. This mammalian mitochondrial enzyme catalyses the initial reaction of the steroid biosynthesis and converts cholesterol to pregnenolone. In this reaction, termed cholesterol side chain cleavage, pregnenolone is produced following three consecutive oxidations of the cholesterol side chain that occur at a single active site on the enzyme [Lambeth et al., 1982]. Cytochrome P450scc is a part of mitochondrial hydroxylation steroid system together with an iron–sulfur protein, adrenodoxin (Adx), and a flavoprotein, adrenodoxin reductase (AdR). CYP11A1 is a membrane-protein associated with the inner membrane of the mitochondrial where it interacts with Adx, a soluble low molecular ferrodoxin-type electron-transfer protein. The reducing equivalents used for the cytochrome P450scc reactions are provided from NADPH via NADPH-AdR and Adx [Hannemann et al., 2001]. Cytochrome P450scc is expressed in the adrenals and gonads, under the control of pituitary peptide hormones, in a hypothalamus–pituitary–adrenal regulatory axis [Chung et al., 1997]. Deficiency in cytochrome P450scc activity can lead to hypertension, feminization, and glucocorticoid insufficiency [Lin et al., 1991]. Moreover, it is known that cytochrome P450scc catalyses the reaction rate-limiting in the synthesis of pregnenolone by human placenta [Tuckey and Cameron, 1993].

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Placental progesterone is essential in the maintenance of pregnancy and its synthesis increases from first trimester of pregnancy until term [Tuckey et al., 2001]. Because of its physiological importance, mitochondrial monooxygense systems, and thereafter the CYP11A1 enzyme have been the focus of many studies [Virckery, 1997; Müller et al., 1998]. In literature, different electron transport models in steroid hydroxylation system have been proposed: shuttle model [Hanukoglu and Jefcoate, 1980], ternary complex model [Kido and Kimura, 1979] and a model with two Adx molecules in electron transfer system [Hara et al., 2000]. In the shuttle model, more supported by scientific study, the Adx is a mobile electron shuttle sequentially binding AdR and then cytochrome P450scc. In the associations between Adx and its redox partners, the electrostatic interactions play an important role, as demonstrated in several studies regarding protein–protein interaction investigated by site-directed mutagenesis [Zöllner et al., 2002]. Few site-directed mutagenesis studies on the expressed bovine enzyme were performed to investigate the hypothetical role of the residues involved in the binding of Adx [Wada and Waterman, 1992; Pikuleva et al., 1995; Woods et al., 1998; Usanov et al., 2002]. Moreover, even if we are working in this direction [Pechkova and Nicolini, 2004; Pechkova et al., 2004], at the moment the 3D structure of cytochrome P450scc has not been resolved. Furthermore the structure–function correlation has not been extensively examined. In an attempt to identify the domain of protein–protein interaction, the role of Asp 405 and Glu124 acidic residues has been investigated in this work. Topological surface analysis of cytochrome P450scc has been performed to support the site-directed mutagenesis study with computer modeling. Two mutants have been realized by site-direct mutagenesis and then characterized by biophysical techniques. Spectral determination and spectroscopic characterization of the cytochrome P450scc mutants permit to monitor possible changes in the 2D structure. Moreover, electrochemical measurements and cholesterol side chain cleavage assays have allowed to obtain the functional characterization of the engineered enzymes. At last, the hypothetical influence of these two mutations in the Adx interaction mechanism has been analyzed by differential spectral titration.

MATERIALS AND METHODS

Protein, Plasmids, Chemicals, and Medium

The expression plasmid pTrc99A-P450scc was obtained as described in Ghisellini et al. [2004]. *Escherichia coli* strains JM109 cells were obtained from Stragene (La Jolla, CA). Reagents for bacterial growth were purchased from Fluka (Buchs, Switzerland). Emulgen 913 was kindly provided by Kao Chemical (Tokyo, Japan). Protein molecular weight standards were obtained from Promega (Madison, WI), the Hydroxyapatite column was from BioRad (Milan, Italy), and the CNBr-activated Sepharose 4B was from Pharmacia (Uppsala, Sweden). Sequencing and mutagenic oligonucleotides were synthesized by TibMolBiol (Genoa, Italy). Recombinant Adx, kindly provided from Prof. R. Bernhardt (Department of Biochemistry, University of Saarlandes, Saarbrucken, Germany), was prepared as described in Uhlmann et al. [1994]. The DEAE-cellulos, HDL, and LDL and other chemicals were purchased from Sigma Chemical Company (Milan, Italy). HDL and LDL are isolated sequentially from plasma by using the modified methods of Rudel et al. [1974] and Burstein and Legmann [1977]. Each lipoprotein is then concentrated and dialyzed extensively against 0.15 M NaCl, 0.01% EDTA, pH 7.4–7.5.

Molecular Modeling of Cytochrome P450scc Mutants

The replacement of the residues proposed for mutations was been performed by InsightII software (Molecular Simulation, Inc., San Diego, CA) running on a Silicon Graphics Crimson (Operating System Irix 6.3). In order to stabilize, the new structures, molecular mechanics (minimizations and dynamics calculations) were performed in the region around the mutation (10 Å). To do that, it was used the program Discover (Molecular Simulation, Inc.). A comparison between mutants and the optimized cytochrome P450scc structure described in Ghisellini et al. [2004] was performed.

Site-Directed Mutagenesis

The cytochrome P450scc mutants were generated by Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Complementary oligonucleotides purified by FPLC were utilized.

TABLE I. Mutagenic Oligonucleotides Used in Side-Directed Mutagenesis Study

Oligonucleotides	Sequences (engineered codon underlined)
Forward	
FAsp405Ile	5′-GGTGGCTGAGTAAAATCAAAGACCTCATCC-3′
FGlu124Ile	5′-GGTGATGGCTCCAATCGCAATAAAGAACTTCATCC-3′
Reverse	
RAsp405Ile	5′-GGATGAGGTCTTTGATTTTACTCAGCCACC-3′
RGlu124Ile	5′-GGATGAAGTTCTTTATTGCGATTGGAGCCATCACC-3′

The sequences of the mutagenic oligonucleotides used to produce the mutations are listed in Table I. The mutants were realized by mutagenesis reaction containing: 5 μl reaction buffer 10×, 10 ng of pTrc99A-P450scc plasmid, 125 ng for each oligonucleotide, 1 μl of dNTPmix 100 mM and 2.5 U of Pfu DNA polymerase in a final volume of 50 μl. After reaction, each sample was treated with a restriction enzyme, Dpn I, and it was used in a transformation reaction with JM109 strain. Plasmids containing the desired mutations were selected by DNA sequencing.

The sequences of the oligonucleotides used for sequencing were: 5′-TGTGTGGAATTGTGAGCG-3′ (scc1f), 5′-CTAGCTGGATTGGTGGAA-3′ (scc1r), 5′-AGTGTCTCAGGACTTCGT-3′ (scc2f), 5′-CTTTCAGGGTATCTCTGC-3′ (scc2r).

Expression and Enzyme Purification

The pTrc99A-P450scc expression plasmid was used to transform JM109 competent cells *E. coli* strains. The heterologous expression of native recombinant (NR) cytochrome P450scc (product of *CYPA11A* gene) was induced by addiction of 1 mM isopropyl-1-thio-β-D-galactopyranoside to exponentially growing cultures according to [Wada et al., 1991] with some modifications: 1 mM δ-aminolevulinic acid, a precursor of heme biosynthesis, was added and the cells were grown for 72 h at 28°C by shaking at 150 rpm. The bacteria obtained from culture were harvested and resuspended in 10 mM Tris-HCl, pH 7.4, containing 0.75 M sucrose. Cells treated with lysozyme (100 μg/ml) were collected by centrifugation and resuspended in 20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 100 μM dithiothreitol. After sonication, the cells were centrifugated at 260,000*g* for 60 min. and *E. coli* membranes were solubilized in 50 mM Tris-HCl, pH 7.4, buffer containing 1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 20% glycerol, and 1% Emulgen 913, for 12 h at 4°C. After centrifugation (105,000*g* for 60 min) in Beckman L-70 Ultracentrifuge, KCl to a final concentration of 50 mM was added to the supernatant and the suspension applied onto a DEAE cellulose column. The flow-through fraction, containing cytochrome P450scc, was dialyzed against buffer A (10 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA, 0.2% sodium cholate and 20% glycerol). Cytochrome P450scc was subsequently loaded onto a hydroxyapatite column (Econo-Pac CHT II) equilibrated with buffer A. After a linear gradient of potassium phosphate (50–300 mM) in buffer, pH 7.4, containing 0.1 mM EDTA, 0.2% sodium cholate and 20% glycerol, was employed to elute the cytochrome P450scc sample. Elution profile was checked spectrophotometrically. The positive fractions (low ratio $A_{280\ nm}/A_{417\ nm}$) were pooled, concentrated, and dialyzed against buffer A. Cytochrome P450scc sample was loaded onto Adx-Sepharose column. The column had been previously equilibrated with buffer A. After an extensive wash with buffer A containing 40 mM KCl the cytochrome P450scc was eluted by buffer A containing 400 mM KCl. Fractions containing purified enzyme were dialyzed against buffer A. The cytochrome P450scc samples were stored at −80°C after adding cholesterol (20 μM). The same purification procedure has been applied for two mutants.

Spectral Determination and Other Analytical Methods

The protein concentration was evaluated by Bradford assay [Bradford, 1976], using bovine serum albumin as a standard. In order to visualize the protein expression or the cytochrome P450scc purified, 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described in Laemmli [1970]. The gel was stained with 0,1% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and properly destained. UV-Vis spectra were recorded using a JASCO 7800

Spectrophotometer (Japan) at room temperature. The native and mutants purified cytochromes P450scc were found to be almost completely in the low spin iron configuration. The heme content was measured according to Omura and Sato [1964] using an extinction coefficient of 91 mM^{-1} cm^{-1} for the absorbance difference between 450 and 490 nm. The purity grade of each sample was performed taking into account the absorbance values at $A_{280\ nm}$ and absorbance maximum at $A_{417\ nm}$ (Soret peak). The absorbance ratios $A_{417\ nm}/A_{280\ nm}$ were >0.9 for all proteins.

Spectroscopic Methods: Circular Dichroism (CD)

CD spectra were carried out using a Jasco 710 spectropolarimeter (Jasco, Osaka, Japan). The instrument was calibrated using (+)-10-camphorsulfonic acid [Tuzimura et al., 1977]. All spectra were recorded in a nitrogen atmosphere at room temperature, using 0.005 cm path-length quartz cell. The data were collected in the far ultraviolet region (180–250 nm) with a wavelength step of 0.2 nm. Each spectrum was a result of an accumulation of ten scans and it was recorded at a rate of 50 nm/min with a time constant of 1 s. The cytochrome P450scc concentration is 0.91 mg/ml in 10 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.2% sodium cholate, and 20% glycerol. The experimental CD spectra were processed to define the contribution of different secondary structure. In order to estimate the secondary structure starting from CD spectra numerous programs are available [Hennessey and Johnson, 1981; Malavalan and Johnson, 1985; Johnson, 1990]. The secondary structure analysis of cytochrome P450scc samples by CD spectra was performed using K2D [Andrade et al., 1993], Contin [Provencher and Glockner, 1981], and Selcon [Sreerama and Woody, 1993] software. In order to evaluate the percentages of α-helices, β-sheet, β-turns, and coils, the conformations were calculated according to the method of Kabsch and Sander [1983].

Differential Spectral Titration of Cytochrome P450scc Mutants

Adx binding to cytochrome P450scc was followed spectrophotometrically by high-spin shift of the P450 heme iron. Spectral titrations were performed as described in Kido and Kimura [1979] using 1 μM of cytochrome P450scc in 10 mM potassium phosphate buffer, pH 7.4, containing, 0.03% Tween-20 and 30 μM cholesterol. The experiments were carried out using tandem cuvettes at room temperature by Jasco 7800 spectrophotometer. The concentrations of free Adx were calculated using the following equation:

$$\mathrm{Adx_{free}} = \mathrm{Adx_{total}} - \left(\frac{\Delta A}{\Delta A_{max}}\right) \times \mathrm{P450scc}.$$

The values of biochemical parameters were determinated by least squared linear regression analysis of the data from three separated experiments.

Electrochemical Measurements and Cholesterol Side Chain Cleavage Assay

The electrochemical measurements were made with a Potentiostat/Galvanostat (EG&G PARC, model 263A) which was supplied with its own software (M270). The working cell was home-made.

The measurements were performed to test the electrochemical response of the binding between the enzymes and their substrates (free cholesterol, HDL, and LDL). The electrochemical analysis with a standard three-electrode configuration was employed where a platinum wire acted as counter electrode and a screen-printed electrodes (s.p.e.) was used as reference and working electrode. The rhodium–graphite s.p.e. were produced using a modified protocol according to Kulys and D'Costa [1991] and Bachmann and Schmid [1999]. The area of working electrode was 2×10 mm. Cyclic voltammetry (CV) experiments were carried out, at room temperature, in 2 ml of 10 mM potassium phosphate buffer, pH 7.4 (working solution) containing 4 μM suspension of enzyme sample. The working electrode was cycled between initial and switch potentials of 500 and −500 mV, respectively, after holding the electrochemical system at the initial potential for 10 s. The 20 mV/s scan rate was used. In order to verify their reproducibility all measurements were repeated three times [Lo et al., 1999; Fernandez-Sanchez et al., 2002]. The chronoamperometric experiments, under convective conditions were performed as a function of the substrate concentration. The cytochrome was completely reduced at a fixed potential of −600 mV versus Ag/AgCl. The free cholesterol (2 mg/ml) was dissolved in 30% sodium cholate, LDL (5.7 mg/ml),

and HDL (11.6 mg/ml) were dissolved in 0.15 M NaCl containing 0.01% EDTA. The experiments with free cholesterol can be performed dissolving it in ethanol or in sodium cholate, but the ethanol denatures the P450 enzymes.

The difference between the cholesterol dissolved in sodium cholate and HDL and LDL is that the first induces only the reorganization of the cholesterol molecules, HDL and LDL are lipoproteins in which the cholesterol is bond to a lot of different proteins.

The electrochemical responses were obtained adding the aliquots of substrates in the working solution (2 ml) every 5 min. The cholesterol side chain cleavage activity of mutants was determinated by HPLC analysis of the reaction products according to the procedures described in Woods et al. [1998]. The product pregnenolone was converted to progesterone by cholesterol oxidase reaction [Sugano et al., 1989].

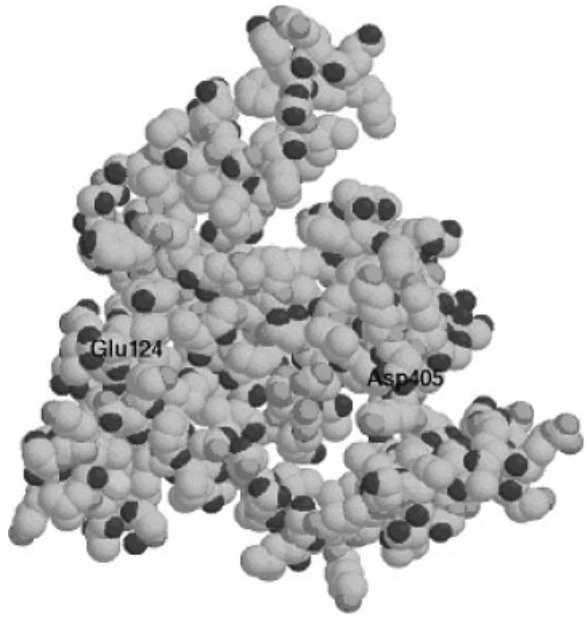

Fig. 1. View on the positive-potential (**left**) facets of the convex hull of the cytochrome P450scc and P450scc atom centers. The residues proposed for mutation are marked.

RESULTS AND DISCUSSION

Topological Surface Analysis of Cytochrome P450scc and Identification of the Residues Involved in Adx Binding

Topological analysis of cytochrome P450scc surface has been performed by applying methods of computational geometry to construct the so-called "convex hull" [Clarkson et al., 1993] of the cytochrome P450scc molecule, i.e., the smallest convex polyhedron containing the molecule. Facets of this convex hull have been analyzed to search for ones with highest electrostatic potential. One such facet with positive potential has been identified. The positive-potential convex hull facet overlaps very strongly with the Adx binding site. This should allow to apply mutagenesis of the same amino acid residues for the optimization of cytochrome P450scc binding to Adx. The negative-potential facet consists of three fragments of P450scc protein structure:

(1) a part of the fragment responsible, according to Vijayakumar and Salerno [1992], to membrane-binding,
(2) a part of the site proposed to be the trypsin-cleavage site,
(3) the C-terminal fragment of cytochrome P450scc.

The positive potential facets are shown in Figure 1. The key factor on which selection of residues for mutagenesis was the following: there are very few hydrophobic residues which allow to introduce additional hydrophobic residues, without possibly promoting the undesirable non-specific aggregation. On the other hand, there are two negatively-charged residues, Asp405 and Glu124, which approach quite closely to the other cytochrome P450scc molecule in the identified complex. Accordingly, removal of those two residues should the Adx binding. The proposed solution is to replace those two residues by hydrophobic residues. In this work, isoleucine residues were analyzed in this respect. The rationale behind this is that, while the isoleucine residue has a large hydrophobic surface area (maximum solvent-exposed area for isoleucine is about 150 $Å^2$), it has less tendency to lose part of this exposure (owing to contacts with neighboring residues) than the phenylalanine or leucine residues in cytochrome P450scc. For instance, out of 30 phenylalanines in P450scc protein, the hydrophobic parts of only two have solvent exposure of more than 100 $Å^2$. For leucines, this number is 9 out of 51, and for isoleucines, 9 out of 32. In terms of energy, 100 $Å^2$ of hydrophobic surface, if removed from contact with solvent during complex formation, adds stabilization to the given configuration of the complex of about 2.5 kcal/mole, which, in turn, corresponds

to increase in the stability constant for the complex of about 50 times. Finally, for each mutation, one has to analyze the effect of that mutation on protein stability. Since the mutations proposed in this work are at the protein surface, they are unlikely to cause changes in the overall fold of cytochrome P450scc. Accordingly, only the effect on residues close enough (within 10 Å) to the mutated residue was analyzed. Of course the optimization methods used do not guarantee that the global energy minimum would be reached. However, basing on the data obtained by denaturation/renaturation of cytochrome P4502B1 [Yu et al., 1995], one can conclude that the cytochrome P450 native structure can be obtained from denatured one only if no extensive unfolding of the protein happens upon denaturation, i.e., the cytochrome P450 structure is, generally speaking, only a local minimum in the conformation space, unlike, for example, cytochrome c that is known to refold into native structure from various denaturated states [McGee and Nall, 1998]. The possible (for cytochrome P4502B1, proven) reason for this difference is that, for cytochromes P450, the global minimum is the very highly aggregated state close to colloid, which becomes reachable if denaturation goes far enough. Since changes of external residues cannot affect the structure of the entire protein, these were obtained by restricted molecular-mechanics optimization of the regions within 10 Å of the mutated residues. The conformation changes are observed only in the residues immediately adjacent to the changed residues while the overall shape of the protein remains the same even in the vicinity of the introduced mutations. To verify that the introduction of the mutants will not damage the protein stability, the same procedure (molecular mechanics optimization restricted to residues within 10 Å of the mutated residue) that was applied to the native protein, and the obtained energies were compared between each of the mutants and the native. As always in molecular mechanics modeling, the absolute values of the energies have no physical significance and are, therefore, not reported here. What is significant is the difference between those values for the mutant and the native proteins. For the mutations, those differences were practically zero, within 0.15 kcal/mol margin. In terms of experimentally observed values (e.g., thermodynamic and kinetic constants), this corresponds to about 30% difference, which is in most cases within the experimental error of their measurement. This suggests that the mutated proteins should be no less thermodynamically stable than the NR cytochrome P450scc.

Mutagenesis and Expression of P450scc Protein

In order to confirm that the site-directed mutagenesis was limited to the predicted sites, cytochrome P450scc cDNA were sequenced using automatic sequencing (Applied Biosystems, Foster City, CA). No undesired mutations have been observed. The native and mutants cytochrome P450scc were expressed as holoproteins in the cytoplasm of *E. coli* cells. The expression yield was calculated estimating the content of cytochome P450scc by CO-difference spectra. In this way, it was evaluated the relationship between the expression condition and the protein synthesis considering possible changes in the folding on the cytochrome (or insertion of the heme) determining the possible presence of inclusion bodies or the inactive form (cytochrome P420). The heme contents per milligram of protein and the spectrophotometric index (A_{416}/A_{280}) have defined the optimal purification protocol for the recombinant cytochrome P450scc. The expression yield of mutated recombinant enzyme in the range of 10 nmol. of the heme/mg protein and A_{416}/A_{280} index was similarly compared to the NR. The final holo-protein index (A_{416}/A_{280}) of

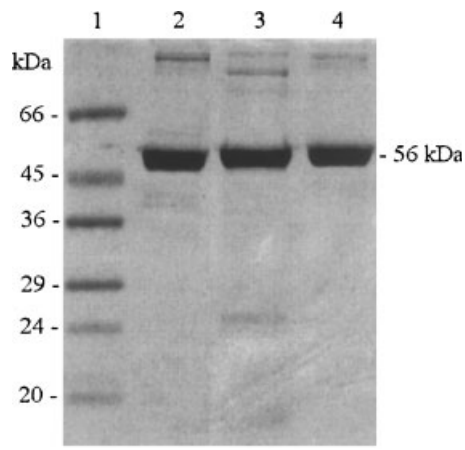

Fig. 2. Ten-percent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the cytochrome P450scc samples. **Lane 1**: Molecular weight markers, (**lane 2**) Glu124Ile mutant, (**lane 3**) Asp405Ile mutant, (**lane 4**) NR cytochrome P450scc. Gel was stained with 0,1% Coomassie Brilliant Blue R-250.

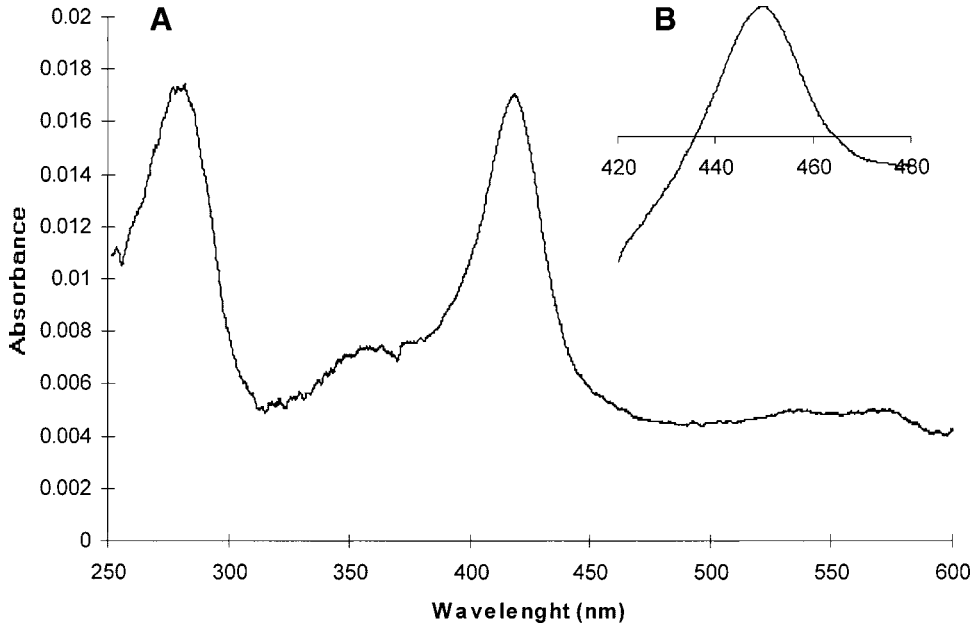

Fig. 3. Optical absorption spectra of oxidized (**A**) and dithionite-reduced CO-bound purified recombinant bovine cytochrome P450scc (**B**). Spectra were recorded in 10 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.2% sodium cholate and 20% glycerol

proteins was equal to 0.90. Each purified sample gave a single protein-staining band (56 kDa) upon analysis by SDS–PAGE (Fig. 2). Figure 3 shows UV-Vis spectrophotometrical characterization of purified mutant Asp405Ile in the oxidized and the dithionite reduced CO complexed states. The oxidized form of the enzyme gave a Soret peak at 417 nm characteristic for low-spin form whereas the dithionite-reduced CO-bound form showed peak at 450 (the inactive form was absent). The spectroscopic analysis of native and Glu124Ile recombinant cytochrome P450scc displayed similar results (data not shown).

Spectroscopic Characterization of the Cytochrome P450scc Mutant

In order to obtain detailed insight into the structural changes in mutated P450scc enzyme we recorded CD spectra in the range from 180 to 650 nm. In Figure 4, CD spectra of the cytochrome P450scc mutants compared with the NR protein were shown. The spectra do not display dramatic changes indicating that the replacement of residues has not cause the secondary structure rearrangements of the native enzyme. The far-UV dichroic spectrum of native and mutants cytochrome P450scc shows two negative bands around 209 and 220 nm and a positive one around 192 nm (Fig. 4); this pattern is typical for proteins with a high content of α-helical secondary structure. The convolution analysis of the CD spectra was performed to estimate the percentage of secondary structure with three different programs (K2D, Selcon and Contin). The obtained data (Table II) show that no major changes occur of the secondary structure of the mutants compared to the native protein.

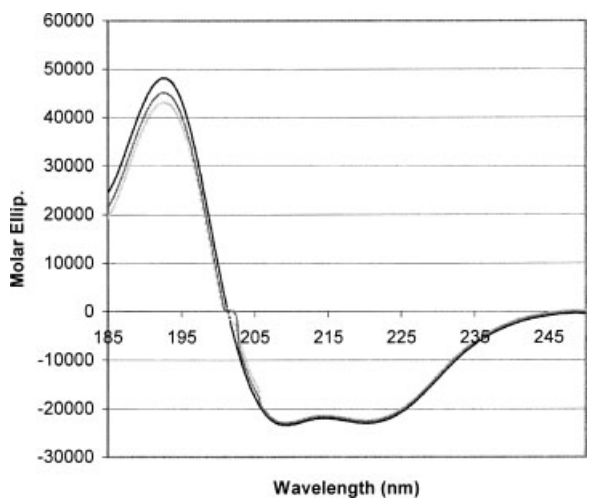

Fig. 4. CD spectra of cytochrome P450scc (light gray) and mutants. CD spectra was recorded in far UV; samples containing 0.91 mg/ml of P450scc protein dissolved in 10 mM potassium buffer, pH 7.4,0.1 mM EDTA, 0.2% sodium cholate and 20% glycerol.

TABLE II. Secondary Structure Prediction of Native and Mutants Cytochrome P450scc

Cytochrome P450scc	Secondary structure[a] (%)		
	α-Helix	β-Sheet	Other
NR	68 ± 2	8 ± 1	24 ± 2
Asp405Ile	73 ± 3	6 ± 1	21 ± 2
Glu124Ile	63 ± 1	7 ± 1	30 ± 3

[a]The secondary structure of the protein was predicted using K2D, Contin and Selcon software.

Spectral Assay of Adx Binding

It was tested whether binding of Adx to cytochrome P450scc is affected by amino acid substitution. The affinity of cytochrome P450scc mutants to Adx was determinated by the analysis of the absorbance changes in the Soret region (393–416 nm) of the cytochrome P450scc which occur upon binding of Adx (Fig. 5). As can be seen in Table III the dissociation constants

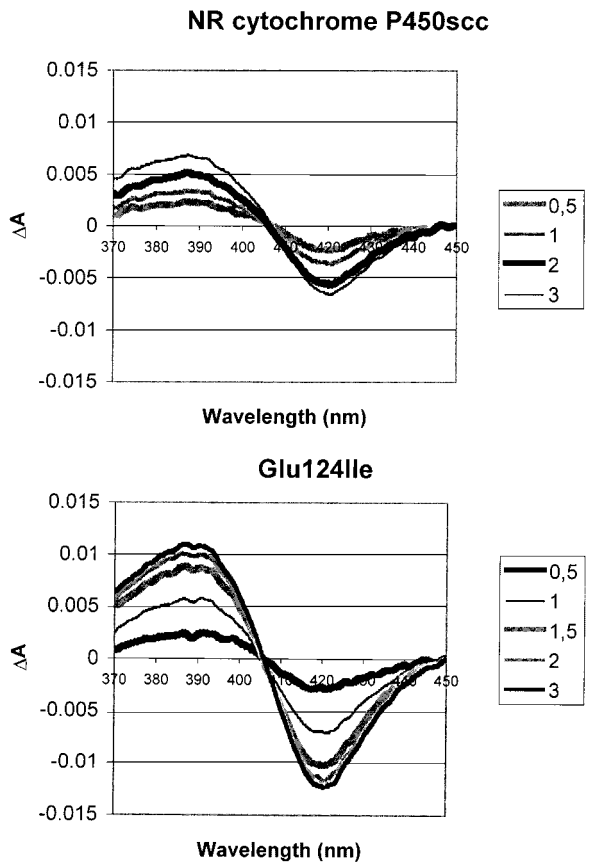

Fig. 5. Spectrophotometric titration of cytochrome P450scc with Adx. Differential spectra obtained from NR (**A**) and mutant Glu124Ile (**B**). Sample containing 1 μM cytochrome P450scc in 10 mM potassium phosphate buffer, pH 7.4, 0.03% Tween-20 and 30 μM cholesterol. The concentrations (μM) of Adx were indicated.

TABLE III. Cytochrome P450scc Spectral Binding Assay

P450scc protein	Dissociation constant for Adx[a] (K_d (μM))
NR	1.05 ± 0.22[b]
Asp405Ile	0.33 ± 0.06[b]
Glu124Ile	0.27 ± 0.06[b]

[a]The apparent dissociation constants for the cytochrome P450scc–Adx complex formation were estimated in 10 mM potassium phosphate buffer, pH 7.4, containing 0.03% Tween-20, 30 μM cholesterol, and 1 μM of cytochrome P450scc.
[b]The standard deviations of dissociation constants were calculated from data of three independent determination.

decrease from 1.05 μM in the NR cytochrome P450scc to 0.33 μM for the Asp405Ile mutant and to 0.27 μM for the Glu124Ile mutant.

Enzymatic Activity of the Cytochrome P450scc Mutants

The cholesterol side chain cleavage activity was determinated for NR and for the mutants. As can to be seen in Table IV there are no significant changes evident for both engineered enzymes.

Cyclic Voltammetric Study

In order to analyze the electrochemical behavior of the engineered cytochromes P450scc, CVs were carried out. The results have been resumed in Table V; where the formal potentials (E'_0), determined as the average of the anodic (E_a) and the cathodic peak (E_c) potentials [$E'_0 = 1/2(E_a + E_c)$] were reported. Usually, E'_0 and the ratio between the electric intensity of the cathodic and the anodic peaks (i_{pa}/i_{pc}) of a redox compound suggest information about the reversibility of the electrochemical process (Nernstian equation) [Churg and Warshel, 1986; Mauk and Moore, 1997; Reipa et al., 2002]. According to the Nernstian equation value, calculated for a monoelectronic redox

TABLE IV. Functional Characteristics of Cytochrome P450scc Enzymes

Cytochrome P450scc	Enzymatic activity assay (% of P450scc NR[a])
NR	100[b]
Asp405Ile	79 ± 3[b]
Glu124Ile	68 ± 2[b]

[a]Cholesterol side chain cleavage activity was initially calculated as nmol of pregnenolone produced/min/nmol of cytochrome P450scc and then the activity of the native recombinant (NR) enzyme was taken at 100%.
[b]Each value represents the average of three experiments.

TABLE V. Electrochemical Parameters Extracted by Cycle Voltammograms of Cytochrome P450scc Proteins

Cytochrome P450scc	E_a (mV)	E_c (mV)	E'_0(mV)	i_{pa} (μA)	i_{pc} (μA)	i_{pa}/i_{pc}
NR	-146 ± 3	-354 ± 3	-208 ± 6	8.89 ± 0.43	11.01 ± 1.10	0.81 ± 0.13
Asp405Ile	-110 ± 1	-333 ± 4	-215 ± 5	9.02 ± 0.45	9.05 ± 1.05	0.94 ± 0.15
Glu124Ile	-108 ± 1	-330 ± 4	-212 ± 4	9.64 ± 0.59	8.42 ± 0.7	1.12 ± 0.17

process in controlled diffusion mechanism (∼−59 mV), the data suggest the irreversibility of the process. The anodic and cathodic peak ratio (i_{pa}/i_{pc}) approaches to 1 in all cases. In literature little $\Delta E'_0$ variations can be found for single aminoacid mutation for metallo-proteins and for active electrochemical groups [Tezcan et al., 1998; Reipa et al., 2002]. Taking into account that, compared with NR cytochrome P450scc the mutants show little $\Delta E'_0$ variations, it is possible to hypothesize that the amino acid replacement does not cause evident changes in the substrate interaction domain.

Chronoamperometry Studies

The chronoamperometry permits to investigate the enzyme-substrate interaction and to estimate the apparent Michaelis–Menten constant (K_M^{app}) [Antonini et al., 2003, 2004]. The K_M^{app} constant can be calculated utilizing this equation [Ortiz de Mantellano, 1986; Xiao et al., 2000; Wang et al., 2002]:

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}}[S],$$

where I_{ss} is the steady-state current after addition of a substrate, [S] is the bulk concentration of the substrate and I_{max} is the maximum current measured. The I_{max}/K_M^{app} ratio was estimated for all the cytochromes to evaluate their affinity with the cholesterol (Table VI).

The little significant variations of I_{max}/K_M^{app} values suggest that the mutations do not influence the enzyme-substrate interaction mechanism. This is probably due to the fact that the substitution of the acidic residues with hydrophobic isoleucines does not cause structural perturbations in the substrate interaction site. Several measurements were performed utilizing the physiological cholesterol forms that it is not free in the blood but bound with different proteins. The behavior of the cytochromes, taking into account the different substrates, was summarized in Table VI. For all samples, the highest I_{max}/K_M^{app} ratio value has been found in the interaction between the free cholesterol and the enzyme. In fact while the cholesterol is free to interact with the enzyme, the cholesterol in HDL and LDL is connected with a lot of different compounds which limit the interaction [Nielsen, 1999; Matsunaga et al., 2002].

CONCLUSIONS

By computer modeling study two residues, potentially involved in the binding between cytochrome P450scc and Adx, were identified. In particular Asp405 and Glu124 residues, located on the positive potential region of cytochrome P450scc, have been candidated to be replaced with hydrophobic residues (two isoleucines). The experimental data obtained by spectrophotometric assay of cytochromes P450scc-Adx interaction have confirmed the computer modeling hypothesis. It is evident that a drastic change in the affinity for Adx was

TABLE VI. Kinetic Parameters of Cytochrome P450s-Substrates Interaction Extracted by Chronoamperometry

	Cholesterol		HDL		LDL	
Cytochrome P450scc	I_{max} (μA), K_M^{app} (μM)	I_{max}/K_M^{app} (μA/mM)	I_{max} (μA), K_M^{app} (μM)	I_{max}/K_M^{app} (μA/mM)	I_{max} (μA), K_M^{app} (μM)	I_{max}/K_M^{app} (μA/mM)
NR	3.67 ± 0.12, 111.23 ± 0.22	33.00 ± 1.14	3.02 ± 0.30, 101.84 ± 0.35	29.65 ± 2.05	1.42 ± 0.15, 107.44 ± 0.55	13.21 ± 1.30
Asp405Ile	4.58 ± 0.12, 100.30 ± 0.31	45.7 ± 1.3	2.68 ± 0.20, 102.37 ± 0.43	26.37 ± 1.92	1.33 ± 0.14, 100.16 ± 0.35	13.27 ± 1.35
Glu124Ile	4.88 ± 0.15, 115.25 ± 0.40	42.3 ± 1.5	2.79 ± 0.17, 102.07 ± 0.23	27.33 ± 1.93	1.12 ± 0.10, 91.44 ± 0.51	12.25 ± 1.05

exhibited by Glu124Ile and Asp405Ile cytochromes P450scc. The obtained K_d values for mutants (Table III) were approximately three fold decreased compared to NR cytochrome P450scc. These results suggest that Asp405 and Glu124 residues seem to be at the cytochrome P450scc-Adx interaction domain or at least close to it (they, of course, could also exhibit an indirect effect).

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