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# Site-Directed Mutagenesis of Cytochrome $b_5$ for Studies of Its Interaction with Cytochrome P450

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**Abstract**—We have shown earlier that microsomal cytochrome $b_5$ can form a specific complex with mitochondrial cytochrome P450 (cytochrome P450scc). The formation of the complex between these two heme proteins was proved spectrophotometrically, by affinity chromatography on immobilized cytochrome $b_5$, and by measuring the cholesterol side-chain cleavage activity of cytochrome P450scc in a reconstituted system in the presence of cytochrome $b_5$. To further study the mechanism of interaction of these heme proteins and evaluate the role of negatively charged amino acid residues Glu42, Glu48, and Asp65 of cytochrome $b_5$, which are located at the site responsible for interaction with electron transfer partners, we used site-directed mutagenesis to replace residues Glu42 and Glu48 with lysine and residue Asp65 with alanine. The resulting mutant forms of cytochrome $b_5$ were expressed in *E. coli*, and full-length and truncated forms (shortened from the C-terminal sequence due to cleavage of 40 amino acid residues) of these cytochrome $b_5$ mutants were purified. Addition of the truncated forms of cytochrome $b_5$ (which do not contain the hydrophobic C-terminal sequence responsible for interaction with the membrane) to the reconstituted system containing cytochrome P450scc caused practically no stimulation of catalytic activity, indicating an important role of the hydrophobic fragment of cytochrome $b_5$ in its interaction with cytochrome P450scc. However, full-length cytochrome $b_5$ and the full-length Glu48Lys and Asp65Ala mutant forms of cytochrome $b_5$ stimulated the cholesterol side-chain cleavage reaction catalyzed by cytochrome P450scc by 100%, suggesting that residues Glu48 and Asp65 of cytochrome $b_5$ are not directly involved in its interaction with cytochrome P450scc. The replacement of Glu42 for lysine, however, made the Glu42Lys mutant form of cytochrome $b_5$ about 40% less effective in stimulation of the cholesterol side-chain cleavage activity of cytochrome P450scc, indicating that residue Glu42 of cytochrome $b_5$ is involved in electrostatic interactions with cytochrome P450scc. Residues Glu42 and Glu48 of cytochrome $b_5$ appear to participate in electrostatic interaction with microsomal type cytochrome P450.

*Key words*: cytochrome $b_5$, cytochrome P450, cytochrome P450scc, site-directed mutagenesis, heterologous expression, protein–protein interactions

Cytochrome $b_5$, a component of endoplasmic reticulum membranes, is a heme protein with molecular weight 16.8 kD; it consists of two functional domains—a hydrophilic heme containing domain that retains all the spectral properties of full-length cytochrome $b_5$ and a short hydrophobic (40 amino acid residues) domain that is responsible for interaction with the microsomal membrane [1, 2]. In erythrocytes, cytochrome $b_5$ occurs in the low molecular weight form that coincides with the hydrophilic fragment of the full-length protein. The full-length and truncated forms of cytochrome $b_5$ are coded by the same gene, the two forms arising at the level of mRNA splicing. The main functional role of cytochrome $b_5$ is to participate in different electron transfer reactions where it serves as a one-electron carrier. Cytochrome $b_5$ plays an important role in such physiologically important reactions as desaturation of fatty acids [1, 3], reduction of methemoglobin to hemoglobin in erythrocytes [4], and hydroxylation of N-acetyl-neuraminic acid [5], and it participates in different reactions of microsomal oxidation catalyzed by various cytochromes P450 [6-8]. Of current interest is the study of the ability of cytochrome $b_5$ to form specific complexes with different types of electron transfer proteins. Cytochrome $b_5$ can form specific complexes in which electron transfer occurs with cytochrome *c* [9, 10], with its physiological electron transfer partner flavoprotein NADH-cytochrome $b_5$ reductase [11, 12], with NADPH-cytochrome P450 reductase [13, 14], and

*Abbreviations*: PCR) polymerase chain reaction; IPTG) isopropyl-β-D-thiogalactopyranoside; PMSF) phenylmethylsulfonyl fluoride; SDS) sodium dodecyl sulfate; HPLC) high pressure liquid chromatography; DHEA) dehydroepiandrosterone.

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different forms of microsomal cytochrome P450 [15-21]. It was recently shown that cytochrome $b_5$ could also interact with mitochondrial type cytochromes P450—cytochrome P450scc [22, 23] and cytochrome P450cam [24]. Cytochrome $b_5$ also plays an important role in regulation of some reactions of steroid synthesis, in particular in regulation of 17α-hydroxylase and 17,20-lyase activities of cytochrome P45017α [25, 26].

The interaction of cytochrome $b_5$ with cytochrome *c* has been studied in greatest detail. Using chemical modification, site-directed mutagenesis, and molecular modeling, it was shown that negatively charged amino acid residues Glu42, Glu48, and Asp65 surrounding the heme of cytochrome $b_5$ and the carboxylic group of the propionic acid of heme may be involved in the interaction with cytochrome *c* [27-31].

To further study the mechanism of interaction of microsomal cytochrome $b_5$ with a mitochondrial type cytochrome P450 (cytochrome P450scc) and to evaluate the role of the negatively charged residues of cytochrome $b_5$ that have been shown to be responsible for the interaction of cytochrome $b_5$ with cytochrome *c* and microsomal cytochrome P450, in the present work we have used site-directed mutagenesis to replace residues Glu42, Glu48, and Asp65 of rat cytochrome $b_5$ with residues with opposite or neutral charge. The glutamate residues Glu42 and Glu48 of cytochrome $b_5$ were replaced with lysine; the aspartate residue Asp65 was replaced with alanine. The results of the present work suggest that glutamate residues Glu42 and Glu48 participate in electrostatic interactions with microsomal cytochromes P4503A4 and P45017α, while glutamate residue Glu42 is more important for interaction with mitochondrial cytochrome P450scc.

## MATERIALS AND METHODS

Isopropyl-β-D-thiogalactopyranoside (IPTG), agarose for electrophoresis, and low temperature melting agarose for electrophoresis were obtained from Gibco BRL (USA), yeast extract, peptone, and tryptone from Difco (USA), cholesterol, pregnenolone, testosterone, 17α-hydroxypregnenolone, sodium cholate, Tween 20, Coomassie G-250, glucoso-6-phosphate, glucoso-6-phosphate dehydrogenase, cholesterol oxidase, polyethylene glycol (6 kD), and 4-(2-hydroxyethyl)-1-piperazine-ethanesufonic acid (HEPES) from Serva (Germany), Sepharose 4B, CNBr-activated Sepharose 4B, DEAE-Sepharose 6B, Sephadexes G-25 and G-75 from Pharmacia (Sweden), δ-aminolevulinic acid, phenylmethylsulfonyl fluoride (PMSF), and NADPH from Sigma (USA), [$^3$H]-17α-hydroxypregnenolone from NEN (USA), TSK-gel HW-50 from Toyopearl (Japan), Bio-Gel HTP from BioRad (USA), Ni-NTA-agarose from QIAGEN (USA), and $Ni^{2+}$ His-Bind from Novagen (USA).

Restriction enzymes and enzymes for DNA modification were obtained from New England Biolabs (England), Promega (USA), and Boehringer (Germany).

**Bacteria and vectors.** The pCR$^{TMII}$ plasmid from Invitrogene (USA) was used to clone PCR products. The His-tag was inserted into the N- or C-terminal sequences of cytochrome $b_5$ using plasmid pQE-32 from QIAGEN (USA) and pCWori$^+$HT, respectively.

All manipulations during cloning and expression of cytochrome $b_5$ mutants were done using *E. coli* JM 109 and BMH-71-18 from Promega (USA) and DH-5α, BL-21 (DE3), and SG 13009 from Stratagene (USA).

The plasmid pSK$^+b_5$, containing cDNA coding the full-length sequence of rat microsomal cytochrome $b_5$, was kindly presented by Prof. A. Ito (Kyushu University, Japan). Expression vector pCWori$^+$ was kindly provided by Prof. M. R. Waterman (Vanderbilt University, USA).

**Site-directed mutagenesis of cytochrome $b_5$.** Site-directed mutagenesis of cytochrome $b_5$ was carried out using an Altered Sites$^{TM}$ System kit (Promega, USA) containing the pSELECT$^{-1}$ vector and helper phages R408 and M13, using the following primers:

Glu42Lys: 5′G ATG CTC TT***T*** GAG AAA CTT 3′
Glu48Lys: 5′G GAC TTC TT***T*** CCC ACC A 3′
Asp65Ala: 5′TG CCC GAC G***G***C CTC AAA GT 3′

Oligonucleotide primers for mutagenesis were synthesized using an Applied Biosystems oligonucleotide synthesizer.

First, the nucleotide sequence containing cDNA coding rat microsomal cytochrome $b_5$ was amplified by using primers 5′ GG*GAATTCCAT ATG* GCT GAA CAA TCA GAT AAA GAT GTG AAG TAC TAC ACT CTG GAG and 3′ GGA CAG ACA GGC TTC GGT TCC *AGA TCT* CG, which allowed us to insert additional restriction sites *Nde* I and *Xba* I as well as to increase the content of AT pairs in the N-terminal region of the cDNA coding cytochrome $b_5$, this preventing the formation of elements of secondary structure of mRNA and supporting high expression level. The amplification products were cloned to the pCR$^{TMII}$ vector and then were re-cloned to the vector for mutagenesis in pSELECT$^{-1}$ using the *EcoR* I site in reverse orientation. The correctness of insertion was proved by the presence of a 450 bp insert after restriction analysis of the plasmid with *Xba* I.

The site-directed mutagenesis of cytochrome $b_5$ was done according to the protocol of the Altered Sites$^{TM}$ System kit and consisted of annealing of mutagenic and selective (repairing the resistance to ampicillin) primers with the single strand of template and synthesis of the second strand with T4 DNA-polymerase with subsequent ligation. The heteroduplex obtained was used to transform *E. coli* BMH-71-18-mut S cells deficient in the reparation system. Mutant forms were selected in medium containing ampicillin. The plasmid DNA was used to

transform *E. coli* JM109. The colonies resistant to ampicillin were selected further by restriction analysis and sequencing, and the positive colonies containing the plasmid with mutation were used to re-clone sequence coding cytochrome $b_5$ to the expression vector.

The presence of the desired mutations in cDNA coding cytochrome $b_5$ was proved by automatic sequencing using an Applied Biosystems A377 DNA sequencer (USA).

**Engineering of expression plasmids.** Plasmid DNA pSELECT$^{-1}$ $b_5$ from the positive clones with proved mutation was treated with fivefold excess of *Nde* I–*Xba* I for 10-15 h. Restriction enzyme *Nde* I was added in portions due to its instability and quick loss of activity. The restriction products were separated by preparative agarose electrophoresis. The corresponding zones were carefully cut out from the gel under UV light, and DNA fragments were purified from the gel using an agarose gel extraction kit from Boehringer with subsequent DNA precipitation with ethanol to remove the remaining chaotropic agent used in the kit that might inhibit ligase activity.

Plasmid DNA for ligation was prepared by treatment of pCWori$^+$ vector with *Nde* I –*Xba* I, and the remainder of the vector was purified by electrophoresis in 0.8% agarose. The purified vector fragment was ligated with the *Nde* I–*Xba* I fragment containing cDNA coding cytochrome $b_5$ or one of its mutants using fivefold excess ligase (New England Biolabs) at 16°C for 5 h. The ligation products were used to transform *E. coli* DH5α. Positive colonies were proved by restriction analysis at *BamH* I–*Xba* I sites to see the presence of the insert.

To facilitate the purification of recombinant cytochrome $b_5$ using metallo-affinity chromatography, we inserted an additional six histidine residues into either the N- or C-terminal sequence of the protein. To insert the His-tag fragment into the N-terminal sequence of cytochrome $b_5$, cDNA coding cytochrome $b_5$ was cloned into plasmid pQE-32. The plasmid pCWori$^+$HT was used to insert the His-tag into the C-terminal sequence of cytochrome $b_5$. To make this re-cloning possible, we introduce an additional *Sal* I site into the C-terminal sequence of cDNA coding cytochrome $b_5$ by PCR amplification using the following primers:

*Sal* I *Nde* I
5′ GGG TCG ACC CAT ATG GCT GAA CAA TCA GAT AAA GAT GTC 3′

*Sal* I
5′ G GAA TTC CGT CGA CTC TTC TGC CAT GTA GAG GCG 3′

**Expression of cytochrome $b_5$ and its mutant forms in *E. coli*.** Expression plasmids containing either cDNA coding cytochrome $b_5$ or its mutant forms were used to transform different *E. coli* cells—DH5α, BL-21, JM109, and SG13009. For analytical expression, an overnight culture was diluted 1 : 100 with TBS medium (1000 ml of medium contains: 12 g yeast extract, 6 g tryptone, 1 g peptone, 2 ml glycerol, 0.17 M $KH_2PO_4$, 0.72 M $K_2HPO_4$) containing 200 μg/ml ampicillin, and the cells were grown at 37°C and 180 rpm until absorbance $A_{600}$ = 0.7-1.0. Then the expression of cytochrome $b_5$ was induced by the addition of IPTG to final concentration 0.5 mM. Temperature and rotation were decreased to 22°C and 120 rpm, respectively, and the culture incubated for an additional 24-48 h. At expression level of cytochrome $b_5$ or its mutants more than 1000 nmol per liter, the best clones can be visualized by intensive pink color. Optimization of the expression procedure allowed us to get from 3000 to 5000 nmol of recombinant cytochrome $b_5$ from 1 liter of culture (12-15 g wet cells), this representing more than 40-50% of total bacterial protein.

**Isolation and purification of cytochrome $b_5$ and its mutant forms from *E. coli* cells.** Recombinant cytochrome $b_5$ was purified as described earlier [32, 33]. After treatment with lysozyme, the cells were sonicated, and membrane and cytosolic fractions of *E. coli* were separated by centrifugation at 100,000*g* for 1 h. The concentration of cytochrome $b_5$ was determined from the absolute absorbance spectrum using molar extinction coefficient $\varepsilon_{413}$ = 117 $mM^{-1}\cdot cm^{-1}$ or from the reduced-minus-oxidized difference spectrum using molar extinction coefficient $\varepsilon_{424\text{-}409}$ = 185 $mM^{-1}\cdot cm^{-1}$, respectively [34]. The purification procedure for cytochrome $b_5$ consisted of several chromatographic steps on DEAE ion-exchange columns. In the final step, we used chromatography on ω-aminooctyl-Sepharose 4B.

The truncated forms of cytochrome $b_5$ and its mutant were purified according to [32] using chromatography on a Mono-Q FPLC column (Pharmacia, Sweden).

Cytochrome $b_5$ with the His-tag at the N-terminal sequence was purified using the following protocol. After disruption of the *E. coli* cells, the membrane fraction (100,000*g* pellet) was solubilized by adding sodium cholate (final concentration 1.5%), and the mixture was incubated for 40 min. Insoluble material was removed by centrifugation at 100,000*g* for 1 h. The supernatant after centrifugation was diluted twofold and applied to a His-Bind Resin column equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, 0.75% sodium cholate, 0.1 mM DTT, and 5 mM imidazole. The column was washed with 10 volumes of equilibration buffer but containing 25 mM imidazole, and cytochrome $b_5$ was eluted in the buffer containing 200 mM imidazole. Cytochrome $b_5$ was applied to a column with thiopropyl-Sepharose 6B (Pharmacia, Sweden) preliminary regenerated with 1.5 mM dipyridyl-disulfide and equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.3 M NaCl and 0.5% sodium cholate. The column was washed with 10 volumes of equilibration buffer, and cytochrome $b_5$ was eluted with the addition of 20 mM DTT to the buffer.

The purification procedure for cytochrome $b_5$ containing the His-tag in the C-terminal sequence consisted of metallo-affinity chromatography on His-Bind Resin, the conditions for which where very similar to those described above, and included ion-exchange chromatography on DEAE-cellulose (DE-52, Whatman, England). The eluate from the metallo-affinity column was 10-fold diluted and applied to the DEAE-cellulose column, equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 0.5% sodium cholate, 0.1 mM EDTA, and 0.1 mM PMSF. Cytochrome $b_5$ was eluted with a 0.1-0.4 M gradient of NaCl in the same buffer.

**Purification of the protein components of the cholesterol side-chain cleavage system.** Cytochrome P450scc, adrenodoxin, and adrenodoxin reductase were purified from bovine adrenocortical mitochondria as previously described [35]. The concentration of cytochrome P450scc was determined from the dithionite reduced carbon monoxide difference spectrum using molar extinction coefficient $\varepsilon_{450\text{-}490} = 91\ mM^{-1}\cdot cm^{-1}$ [36]. The concentrations of adrenodoxin and adrenodoxin reductase were determined from absolute absorbance spectra using molar extinction coefficients $\varepsilon_{414} = 10.0\ mM^{-1}\cdot cm^{-1}$ and $\varepsilon_{450} = 11.3\ mM^{-1}\cdot cm^{-1}$, respectively [37].

**Purification of recombinant cytochromes P4503A4 and P45017α from *E. coli* cells.** Cytochromes P4503A4 and P45017α were expressed in *E. coli* cells as derivatives containing six histidine residues in the C-terminal sequence allowing the use of metallo-affinity chromatography for their purification. Membranes prepared from sonicated recombinant *E. coli* cells were suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol and 50 μM testosterone when purifying cytochrome P4503A4, or 50 μM progesterone when purifying cytochrome P45017α. Cytochrome P450 was solubilized by addition to the membrane suspension of 10% Emulgen 913 to final concentration 1% (approximately 3 mg Emulgen 913 per mg protein). The suspension was stirred for 1 h on ice at 4°C and then centrifuged at 100,000*g* for 30 min. The supernatant containing solubilized cytochrome P450 was applied to a Ni-NTA-agarose column. For the purification of cytochrome P4503A4, a precolumn with DEAE-cellulose was used to remove some impurities that compete with cytochrome P4503A4 for binding with the affinity matrix. The column was washed with 10 volumes of 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 100 mM glycine, 0.3 M NaCl, and 0.2% Emulgen 913, and the cytochrome P450 was eluted with the same buffer but containing 50 mM histidine.

The fractions containing cytochrome P450 were collected and diluted fourfold with 20% glycerol containing 0.1 mM dithiothreitol and 50 μM testosterone or progesterone for cytochrome P4503A4 or P45017α, respectively, and applied to a hydroxyapatite column (1.5 × 10 cm). The column was washed with five volumes of 50 mM sodium phosphate buffer, pH 7.2, containing 20% glycerol and 0.1 mM dithiothreitol, and cytochrome P450 was eluted by increasing the concentration of phosphate buffer to 300 mM. Cytochromes P4503A4 and P45017α were dialyzed against 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol and 0.1 mM dithiothreitol and stored at −70°C.

**Analytical methods.** The protein composition of bacterial cells after protein expression and the purity of purified proteins monitored by SDS-PAGE in 12% gel according to Laemmli [38] using a mini Protean II electrophoresis system (BioRad, USA). The recombinant proteins were identified by immunoblotting [39]. To determine the concentration of cytochrome $b_5$, cytochrome P450scc, adrenodoxin reductase, and adrenodoxin, the following molar extinction coefficients were used: $117\ mM^{-1}\cdot cm^{-1}$ at 413 nm [40], $91\ mM^{-1}\cdot cm^{-1}$ at 450 nm [36], $11\ mM^{-1}\cdot cm^{-1}$ at 450 nm, and $10\ mM^{-1}\cdot cm^{-1}$ at 414 nm, respectively [37].

**Spectral characterization of the mutant forms of cytochrome $b_5$.** Spectrophotometric measurements were carried out using a Shimadzu UV-3000 (Shimadzu, Japan) spectrophotometer. The concentration of cytochrome $b_5$ was determined from the dithionite-reduced-minus-oxidized difference spectrum using molar extinction coefficient $185\ mM^{-1}\cdot cm^{-1}$ at $A_{424\text{-}409}$. Difference spectra were used to monitor the level of expression of cytochrome $b_5$ in *E. coli* cells and to determine its concentration during the initial steps of the purification procedure.

Circular dichroism spectra were recorded with a JASCO J-20 (JASCO, Japan) spectropolarimeter using the following experimental conditions: optical bandwidth, 1 nm; response time, 2 sec; scan speed, 20 nm/min; temperature, 20°C; averaging over six scans. In the ultraviolet region (200-260 nm), the spectra were measured with optical pathlength 0.5 mm and cytochrome $b_5$ concentration 20.5-30.4 μM in 20 mM Tris-HCl buffer, pH 7.6, containing 0.25% sodium cholate. In the visible region (300-600 nm), cells with 10 mm optical pathlength were used. Molar ellipticity [Θ] was calculated using the equation:

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

where Θ is ellipticity (millidegrees), $C$ protein concentration (M), and $l$ optical pathlength (cm).

The contents of elements of secondary structure of cytochrome $b_5$ (%) were calculated from the circular dichroism spectra in ultraviolet region using computer programs Dichropro 2.5 or CD Spectra Deconvolution 2.1.

**Spectral studies of the interaction of truncated forms of cytochrome $b_5$ with cytochrome *c*.** Cytochrome *c* from horse heart (Serva, Germany) was additionally purified from oligomeric forms on CM-cellulose (CM-52, Whatman). The concentration of cytochrome *c* was

determined from the absolute absorbance spectrum using molar extinction coefficient $\varepsilon_{410} = 106\ mM^{-1} \cdot cm^{-1}$ [40]. For spectral titration experiments, two pairs of matched cells (Hellma, Germany) with optical pathlength 10 mm were used.

The formation of complexes between heme proteins was registered as an increase in absorbance in the difference spectrum in the Soret region as earlier described [40]. Cytochrome $b_5$ was titrated with cytochrome *c* in 2.5 mM phosphate buffer, pH 7.4, at 25°C. After addition of cytochrome *c*, the heme proteins were mixed and incubated 10 min, and then the spectrum was recorded in the region 360-500 nm. The concentration of cytochrome $b_5$ in titration experiments was ~2.0 μM; the concentration of cytochrome *c* was varied in the range 0.4-12.0 μM.

**Determination of cholesterol side-chain cleavage activity of cytochrome P450scc.** Cholesterol side-chain cleavage activity of cytochrome P450scc was determined according to [41]. An HPLC apparatus (Altex, USA) with normal-phase column (Ultrasphere SI, 4.6 × 250 mm) was used with the system *n*-hexane–isopropanol (3 : 1), monitoring at 240 nm the amount of progesterone formed as a result of transformation of reaction product pregnenolone to progesterone in the presence of cholesterol oxidase. The product peaks were analyzed using a Chromatopac C-R1B integrator (Shimadzu, Japan). Deoxycorticosterone was used as an internal standard.

The reaction of cholesterol side-chain cleavage as catalyzed by cytochrome P450scc was carried out in 0.5 ml of 50 mM phosphate buffer, pH 7.4, containing 0.05% Tween 20 in the presence of 25 μM cholesterol and deoxycorticosterone as internal standard. The concentration of cytochrome P450scc, adrenodoxin, and adrenodoxin reductase in the reconstituted system was kept constant at 0.5, 1.0, and 0.5 μM, respectively. Cytochrome $b_5$ and it mutant forms were added to the incubation mixture at final concentration 0.25-3.0 μM, and the proteins were incubated 10 min at room temperature. The reaction was started by addition of an NADPH-regenerating system, and samples were incubated 5 min at 37°C. The reaction was stopped by addition of 200 μl methanol, and the tubes were placed on ice. To convert pregnenolone formed to progesterone, samples were additionally incubated for 10 min at 37°C with cholesterol oxidase (0.12 IU per tube) in 20 mM phosphate buffer containing 0.3% sodium cholate. Steroids were extracted with 2 ml *n*-hexane and evaporated under argon. The mobile phase for HPLC consisted of a mixture of *n*-hexane–isopropyl alcohol at ratio 82 : 18, which was used to dissolve the dried samples before chromatography. Cholesterol side-chain cleavage activity of cytochrome P450scc was determined by integrating the area of the peaks corresponding to the internal standard and the reaction product.

**Determination of 17,20-lyase activity of cytochrome P45017α.** The hydroxylation of 17α-hydroxypregnenolone by cytochrome P45017α with formation of dehydroepiandrosterone (DHEA) was carried out in a reconstituted system at 37°C in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$. Highly purified recombinant bovine cytochrome P45017α containing six histidine residues at the C-terminal sequence was added to incubation mixture to final concentration 0.5 μM. [$^3$H]17α-hydroxypregnenolone was added together with unlabeled steroid dissolved in ethanol to final concentration 50 μM with specific radioactivity 100,000 cpm per sample. The reaction was started by adding NADPH to final concentration 0.5 mM. Aliquots of 0.5 ml were taken from the incubation mixture at selected times and mixed with 5 ml dichloroethane. The mixture was vigorously shaken, and the organic and water phases were separated by centrifugation. The organic layer was carefully removed and evaporated under nitrogen. The steroids were dissolved in 100 μl of methanol and analyzed by HPLC using a computerized Waters 840 instrument equipped with optical flow detector Spectraflow 757 and flow radioactive counter β-RAM, using a 10-μm $C_{18}$ Bondopak column (39 × 300 mm). The reaction products were identified based on the elution time of standards.

**Determination of testosterone 6β-hydroxylase activity of recombinant cytochrome P4503A4.** An aliquot of highly purified recombinant cytochrome P4503A4 (25.5 μl of 39.3 μM) and recombinant NADPH-cytochrome P450 reductase (65.8 μl of 30.4 μM) were added to dioleoylphosphatidylcholine (DOPC, 15 μl of solution, 20 mg/ml) and CHAPS (10 μl of solution, 50 mg/ml) with subsequent addition of different amounts of cytochrome $b_5$ or its mutant form. The reaction mixture was incubated 10 min at 37°C with stirring and then diluted to 2 ml with 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, testosterone, [$^{14}$C]testosterone, and the NADPH-regenerating system. Final concentrations of the components of the incubation mixture were 0.5 μM cytochrome P4503A4, 1 μM NADPH-cytochrome P450 reductase, 0.25 mg/ml CHAPS, 0.15 mg/ml DOPC, 100 μM testosterone (~600,000 cpm for [$^{14}$C]testosterone), 8 mM sodium isocitrate, and 0.1 IU/ml isocitrate dehydrogenase.

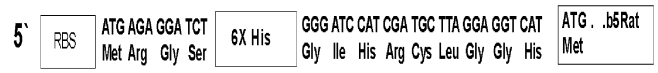
The reaction mixture was incubated 5 min at 37°C and the reaction started by adding NADPH (final concentration 1 mM). Aliquots of 0.5 ml were taken from the incubation mixture every 2.5 min and extracted with 5 ml of methylene chloride. The organic layer was dried under nitrogen, and the steroids were dissolved in 100 μl of methanol and analyzed by HPLC in the same way as described for determination of 17α-hydroxylase activity of cytochrome P45017α.

**Immobilization of cytochrome P450scc.** Cholesterol side-chain cleaving cytochrome P450scc was immobilized on CNBr-activated Sepharose 4B (Pharmacia, Sweden) according to the manufacturer's protocol. The matrix contained approximately 100 nmol of cytochrome P450scc per ml gel.

## RESULTS

**Plasmid constructs used for expression and mutagenesis of cytochrome $b_5$.** The scheme describing the engineering of the constructs for expression and site-directed mutagenesis of cytochrome $b_5$ is shown in Fig. 1. The sequence coding rat microsomal cytochrome $b_5$ in plasmid $pSK^+b_5$ was PCR amplified to introduce extra restriction sites *Nde* I and *Xba* I necessary for subsequent cloning into expression vector $pCWori^+$ and to increase the content of AT pairs in the N-terminal sequence of the cDNA coding cytochrome $b_5$. The product of the PCR reaction was re-cloned from the $pCR^{TMII}$ vector using restriction sites *Nde* I and *Xba* I into $pCWori^+$ vector for expression of the wild-type protein and using the *EcoR* I site into mutagenesis vector $pSELECT^{-1}$. The correctness of orientation was proved by the presence of a 450 bp insert after treatment of the vector with *Xba* I.

To insert an extra six histidine residue tag into the N-terminal sequence of cytochrome $b_5$, cDNA coding wild-type cytochrome $b_5$ was cloned using *BamH* I–*Pst* I restriction sites into vector pQE-32. Since the *BamH* I restriction site in vector $pCWori^+b_5$ is in front of the starting codon, and, in addition, after the pQE-32 promoter there is a translated region coding several amino acids, the final construct contains at the N-terminal sequence an additional linker consisting of 19 amino acids including six histidine residues and a unique cysteine residue:




The plasmid $pCWori^+HT$ was used to introduce a six histidine residue tag into the C-terminal sequence of cytochrome $b_5$. To insert an extra *Sal* I site in the C-terminal sequence of the cDNA coding wild-type cytochrome $b_5$, plasmid $pSK^+b_5$ was preliminary PCR amplified. After cloning of the product of PCR amplification into $pCWori^+HT$ vector using *Nde* I and *Sal* I sites, the final construct coded cytochrome $b_5$ containing the additional sequence -*Ser-Thr-6His* before the terminator of transcription. During these manipulations, the final amino acid residue of cytochrome $b_5$, Asp134, was replaced with Glu, with retention of the negative charge of this residue.

The mutant forms of cytochrome $b_5$ were re-cloned from $pCWori^+$ into $pCWori^+HT$ vector using the internal *Sac* I restriction site of cytochrome $b_5$ and vector site *EcoR V*.

**Expression and purification of mutant forms of cytochrome $b_5$.** The expression conditions and purification protocol for cytochrome $b_5$ mutant forms did not significantly differ from the condition used for expression and purification of wild-type cytochrome $b_5$ [32, 33]. Optimization of the expression procedure allowed reaching preparative scale yields from 3000 to 5000 nmol of recombinant cytochrome $b_5$ per liter of culture (12-15 g wet cells), this representing 40-50% of the total bacterial protein.

At expression levels of cytochrome $b_5$ more than 1000 nmol per liter of culture, the best clones could be visualized by the intense pink color of the recombinant cells without using sensitive methods (SDS-PAGE with subsequent immunoblotting), especially when using *E. coli* DH5α cells. In contrast to wild-type cytochrome $b_5$, the ratio of full-length and truncated forms in preparations of the mutant forms of the heme protein was reversed (see table). The purification of the full-length form of cytochrome $b_5$ required separation of membrane and cytosolic fractions of *E. coli* cells by centrifugation at 100,000*g*. Practically all proteolytically modified cytochrome $b_5$ was found in the cytosolic fraction. During purification of the Glu48Lys and Asp65Ala mutants of cytochrome $b_5$, we noticed an increased hydrophobicity and tendency for aggregation for these mutants as compared to the wild-type cytochrome $b_5$ even in the presence of relatively high detergent concentrations (0.5% sodium cholate) and some decrease in the interaction strength with ion-exchange resin during purification due to partial loss of the negative charge.

The homogeneity of purified cytochrome $b_5$ and its mutant forms was demonstrated by SDS-PAGE (Fig. 2). The purified heme proteins have spectrophotometric index $A_{412}/A_{280}$ of 2.2-2.5 for full-length and 4.6-5.0 for the truncated forms of cytochrome $b_5$, respectively. During purification of some cytochrome $b_5$ mutants, we noticed that despite electrophoretic homogeneity these mutants had decreased spectrophotometric index. We found later using agarose electrophoresis that this was connected with the presence of some low molecular weight RNA. Therefore, the final preparations of highly purified cytochrome $b_5$ were additionally treated with RNase A (40 μg/ml, 37°C, 30 min). The remaining of RNase was removed by passing the cytochrome $b_5$ through a column with CM-52 CM-cellulose.

**Isolation and purification of recombinant cytochrome $b_5$ with modified N- and C-terminal sequences.** One approach simplifying the purification procedure of recombinant proteins is insertion of a tag containing four or six histidine residues into either the N- or C-terminal sequence of the protein, thus allowing the use of metallo-affinity chromatography [42-45]. However, sometimes during expression of membrane-bound heme proteins one can see disturbance of the folding process and as a result the apo-form of the heme protein is predominantly expressed [44].

During optimization of expression conditions for recombinant cytochrome $b_5$ containing the His-tag in the N-terminal sequence, the best results were obtained when using *E. coli* JM109 cells, where expression level up to 6000 nmol of cytochrome $b_5$ per liter of culture could be reached. However, despite high expression level, it was

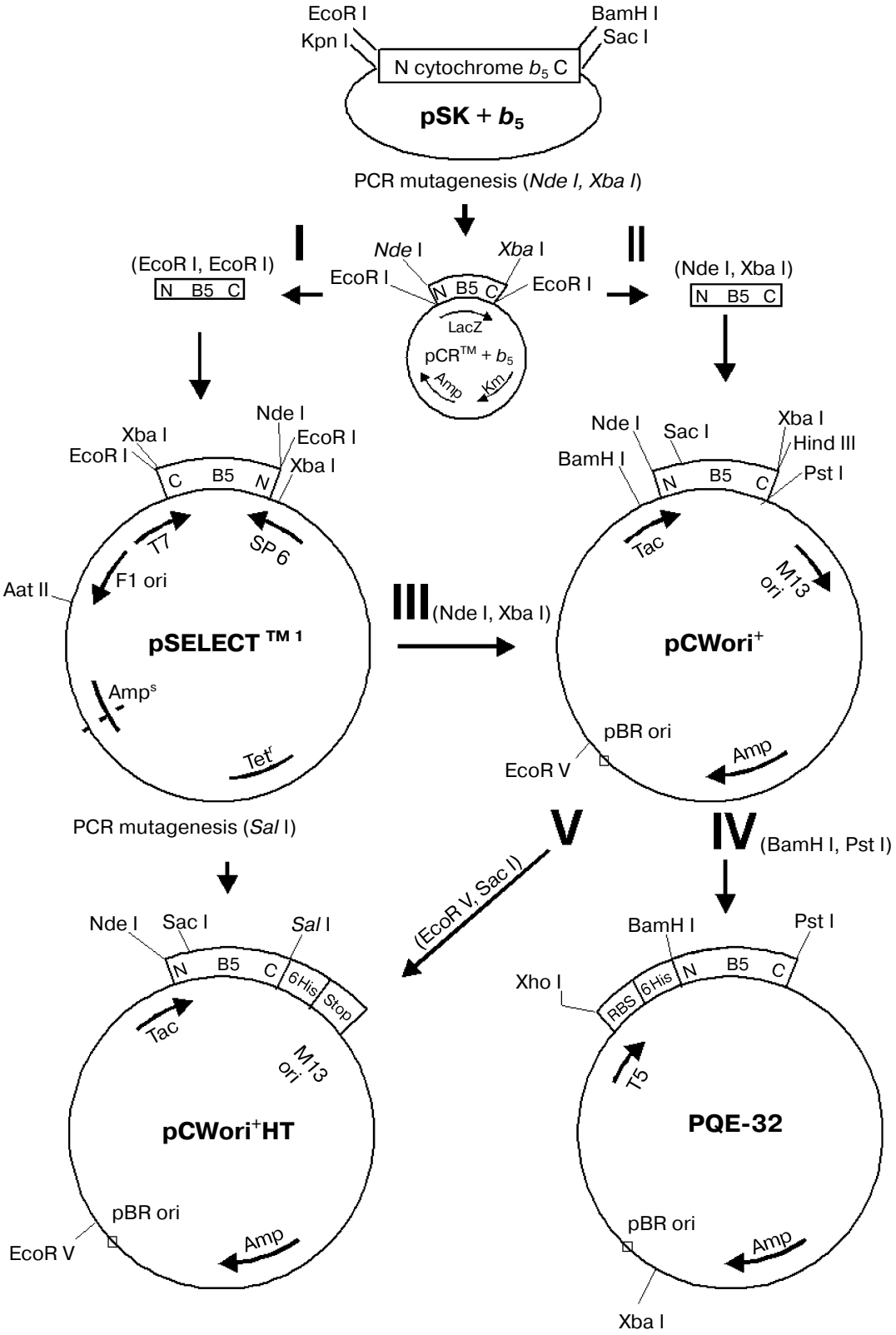



**Fig. 1.** Scheme showing the construction of vectors for mutagenesis and expression of rat cytochrome $b_5$ in *E. coli*. I) Cloning of cDNA of cytochrome $b_5$ into vector for mutagenesis using Altered Sites™; II) cloning of cDNA of wild-type cytochrome $b_5$ and its mutants into expression vector (III); IV) engineering of the construct with additional His-tag in the N-terminal sequence of cytochrome $b_5$; V) cloning of cytochrome $b_5$ mutants into the vector containing C-terminal His-tag.

Purification of full-length recombinant cytochromes $b_5$

| Content of cytochrome $b_5$, nmol | Wild type | Asp65Ala | Glu42lys | Glu48Lys |
|---|---|---|---|---|
| Cells (*E. coli* DH5α) | 2750 | 2650 | 1880 | 1750 |
| Supernatant 100,000*g* (low molecular weight form) | 480 | 1783 | 1160 | 1150 |
| Solubilized membrane fraction (full-length form) | 2220 (100%) | 860 (100%) | 720 (100%) | 690 (100%) |
| Step I (DEAE-cellulose) | 1300 (58%) | 350 (41%) | 330 (46%) | 470 (68%) |
| Step II (DEAE-agarose) | 830 (37%) | 220 (26%) | 180 (25%) | 263 (38%) |
| Step III (ω-aminooctyl-Sepharose 4B) | 720 (32%)<br>58 nmol/mg | 125 (14.5%)<br>56.7 nmol/mg | 115 (16%)<br>54.3 nmol/mg | 200 (28%)<br>57.3 nmol/mg |

Note: The numbers presented in this table represent results of purification of recombinant cytochrome $b_5$ from 1 liter of incubation culture. The amounts of cytochrome $b_5$ at different steps of the purification procedure are given in nmol.

not possible to purify cytochrome $b_5$ by metallo-affinity chromatography to the homogeneous state in one step since the modified heme protein becomes more hydrophobic. Moreover, due to proteolysis resulting in removal of 40 residues from the C-terminal sequence of cytochrome $b_5$, both full-length and truncated forms interact with the metallo-affinity column. Therefore, for purification of the full-length form of cytochrome $b_5$ we used only the membrane fraction of recombinant *E. coli* cells. The presence of cytochrome $b_5$ in a fraction that does not bind to the metallo-affinity column indicates limited proteolysis in the N-terminal sequence of cytochrome $b_5$, which removes the His-tag and disturbs the interaction of the cytochrome $b_5$ with the metallo-affinity column.

Immunoblotting analysis confirmed the presence of proteolytically modified forms of cytochrome $b_5$ in *E. coli* cells, which made the use of protease inhibitors ineffective. Since during cloning of cytochrome $b_5$ into the pQE-32 vector we introduced a cysteine residue into the N-terminal sequence of cytochrome $b_5$, which is normally absent in the wild-type protein, we used thiopropyl-

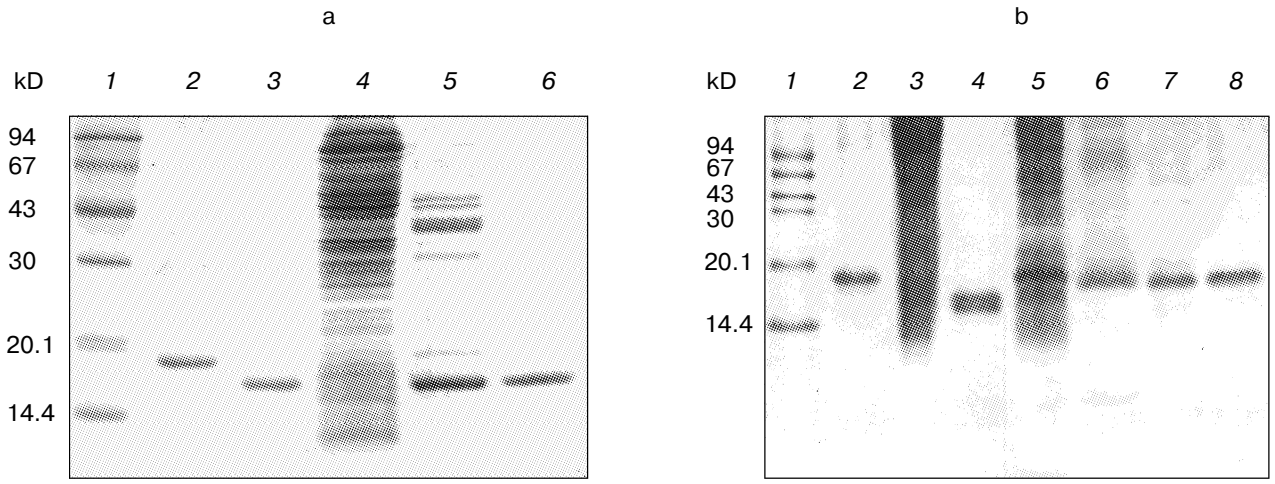



**Fig. 2.** SDS-PAGE (15% gel) of recombinant cytochrome $b_5$ and its mutant forms. a) Purification of low molecular weight cytochrome $b_5$ (Asp65Ala): *1*) 14-94 kD markers; *2*, *3*) full-length and low molecular weight forms of wild-type cytochrome $b_5$; *4*) cytosolic fraction of recombinant *E. coli* DH5α; *5*) low molecular weight form of cytochrome $b_5$ after three steps of purification; *6*) FPLC on MONO-Q; b) purification of full-length cytochrome $b_5$ (Glu48Lys): *1*) 14-94 kD markers; *2*) full-length form of cytochrome $b_5$; *3*) cytosolic fraction of recombinant *E. coli*; *4*) low molecular weight form of cytochrome $b_5$ after three steps of purification; *5*) solubilized membrane fraction of *E. coli*; *6-8*) three step purification of full-length cytochrome $b_5$.

Sepharose 6B to further purify this protein. Using this approach we were able to purify full-length cytochrome $b_5$, but the yield was only 10% of the spectrally detected cytochrome $b_5$ in the solubilized membrane fraction.

More effective was insertion of a His-tag into the C-terminal sequence of cytochrome $b_5$. In this case, metallo-affinity chromatography allowed us to completely remove the truncated form of the heme protein during the first purification step. Cytochrome $b_5$ eluted from the metallo-affinity column is characterized by spectrophotometric index $A_{412/280}$ = 1.1-1.4. The subsequent use of ion-exchange chromatography on DEAE cellulose produced electrophoretically homogeneous cytochrome $b_5$ with spectrophotometric index $A_{412/280}$ = 2.3.

However, after re-cloning Asp65Ala, Glu48Lys, and Glu42Lys mutants of cytochrome $b_5$ into pCWori$^+$HT vector with subsequent expression of the recombinant proteins in *E. coli*, we could not detect the full-length forms of these cytochrome $b_5$ mutants in any subcellular fraction. Insertion of the mutation and an additional His-tag makes the mutant forms of cytochrome $b_5$ more sensitive to posttranslational proteolytic modification. Thus, we returned to the original expression system of the mutants of cytochrome $b_5$ using the pCWori$^+b_5$ vector.

**Spectral characterization of the mutant forms of cytochrome $b_5$.** Absolute absorbance spectra of recombinant wild-type cytochrome $b_5$ and its mutant forms (Fig. 3) having absorbance maximum in the Soret region at 412.5-413 nm for the oxidized heme proteins are identical to the spectra previously described [32, 33]. As in the case of wild-type cytochrome $b_5$, absolute absorbance spectra of mutant forms of cytochrome $b_5$ at the first step of the purification procedure correspond to the reduced form of the heme protein (Fig. 3a) with a shift of the absorbance maximum to 424 nm and appearance of additional peaks at 527 and 557 nm. Highly purified cytochrome $b_5$ has spectrophotometric index $A_{412}/A_{280}$ = 2.2-2.5 for full-length and 4.6-5.0 for the truncated forms of the protein, respectively (Fig. 3b).

Circular dichroism (CD) spectra of recombinant cytochrome $b_5$ and its mutant forms in the far ultraviolet region (200-250 nm) show a negative extreme of molar ellipticity at 208 nm (Fig. 4). After replacement of glutamate at position 48 for lysine (Glu48Lys), the amplitude of the negative molar ellipticity becomes less compared to the wild-type cytochrome $b_5$, indicating some decrease in the degree of packing of the molecule. The content of $\alpha$-helixes according to calculation is decreased by 10% compared to the wild-type protein. This is in accordance with increased sensitivity of cytochrome $b_5$ mutant forms to endogenous proteolytic modification (table).

**Interaction of the truncated form of cytochrome $b_5$ and its mutant forms with cytochrome *c*.** Complex formation between cytochrome *c* and cytochrome $b_5$ is rather simply registered based on changes in the absorbance maximum at 416 nm in the Soret region at low ionic

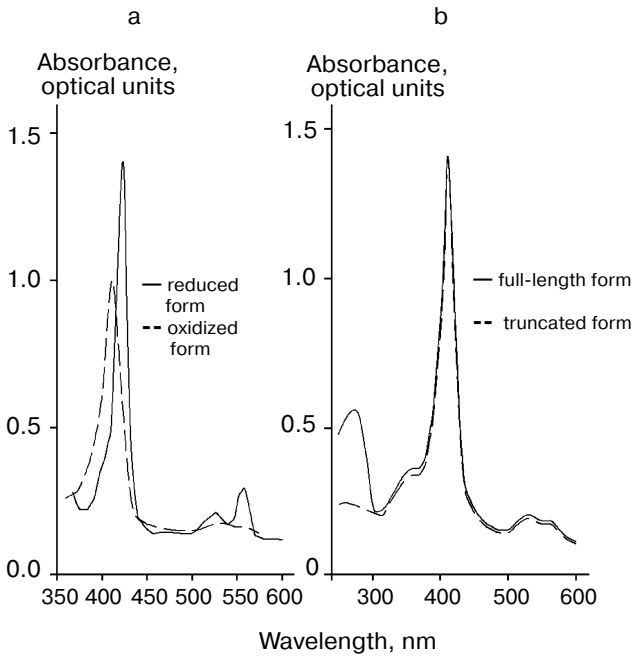



**Fig. 3.** Absolute absorbance spectra of recombinant wild-type cytochrome $b_5$ and its mutant forms. a) Absorbance spectra of cytosolic and solubilized membrane fractions of recombinant *E. coli* after heterologous expression of different types of cytochrome $b_5$; b) absorbance spectra of oxidized forms of purified preparations of full-length and low molecular weight forms of cytochrome $b_5$ (cytochrome $b_5$ concentration ~10 $\mu$M in 20 mM Tris-HCl buffer, pH 7.8, containing 0.25% sodium cholate).

strength ($\mu \leq 50$ mM) and is a very convenient method to evaluate the affinity of these heme proteins after replacement of some amino acid residues of the "acidic" cluster of cytochrome $b_5$ [30, 31]. Data of spectrophotometric titration of cytochrome $b_5$ with cytochrome *c* are presented in Fig. 5. The values of dissociation constants of the complex of the two heme proteins were $(0.66 \pm 0.15)\cdot10^{-6}$, $(1.84 \pm 0.5)\cdot10^{-6}$, $(1.66 \pm 0.14)\cdot10^{-6}$, and $(1.25 \pm 0.2)\cdot10^{-6}$ M for wild-type cytochrome $b_5$ and mutant forms Glu48Lys, Asp65Ala, and Glu42Lys, respectively. The slightly higher values of dissociation constants as compared to the literature data appear to be connected with higher ionic strength ($\mu$ = 2.5 mM instead of the commonly used 1-0.5 mM) used in the titration experiments. According to previous experiments, the stability of the complex between cytochromes *c* and $b_5$ is dramatically decreased at increased ionic strengths, indicating that electrostatic interactions play an important role in complex formation [40]. However, the use of low ionic strength is problematic due to low buffer capacity of potassium phosphate used for titration experiments. The data obtained in this way show a tendency for decreasing cytochrome $b_5$ affinity to cytochrome *c* after replacement of negatively charged

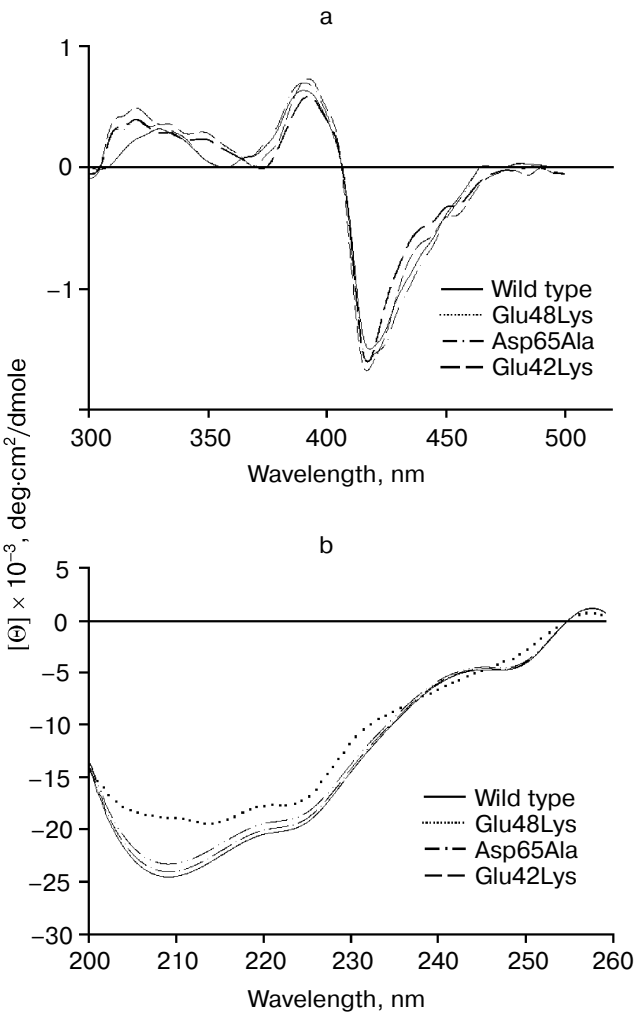



**Fig. 4.** Circular dichroism spectra of recombinant wild-type cytochrome $b_5$ and its mutant forms in the visible (a) and ultraviolet regions (b). Concentration of wild-type and mutant protein is 20-30 μM (20 mM Tris-HCl buffer, pH 7.6, 0.25% sodium cholate) and optical pathlength is 1 and 0.05 cm in the visible and ultraviolet region, respectively.

residues surrounding the heme group of cytochrome $b_5$, indicating their possible involvement in electrostatic interaction with positively charged residues of cytochrome *c*.

**The interaction of cytochrome $b_5$ and its mutants with immobilized cytochrome P450scc.** To evaluate the mechanism of interaction of wild-type cytochrome $b_5$ with cytochrome P450scc and the effect of replacement of amino acid residues from the "acidic" cluster on this interaction using affinity chromatography, we immobilized cytochrome P450scc via amino groups on CNBr-activated Sepharose 4B. We showed earlier that the two heme proteins have high affinity for each other [22, 23]. For that purpose, we used the approach consisting of immobilization of cytochrome $b_5$ [32]. In the present work, chromatography was carried out in 20 mM phosphate buffer, pH 7.4. To the column (0.5 × 1.5 cm) with immobilized cytochrome P450scc, 3 nmol of recombinant wild-type or mutant form of cytochrome $b_5$ was applied. To estimate the affinity to cytochrome P450scc, we used both the full-length and C-terminal-truncated forms of cytochrome $b_5$. The proteins were eluted from the column with a step gradient of ionic strength or using a gradient of NaCl and sodium cholate in the range 0.1-1.0 M and 0.1-0.3%, respectively. The dissociation of the complex occurs only at high ionic strength in the presence of detergent, indicating that both electrostatic and

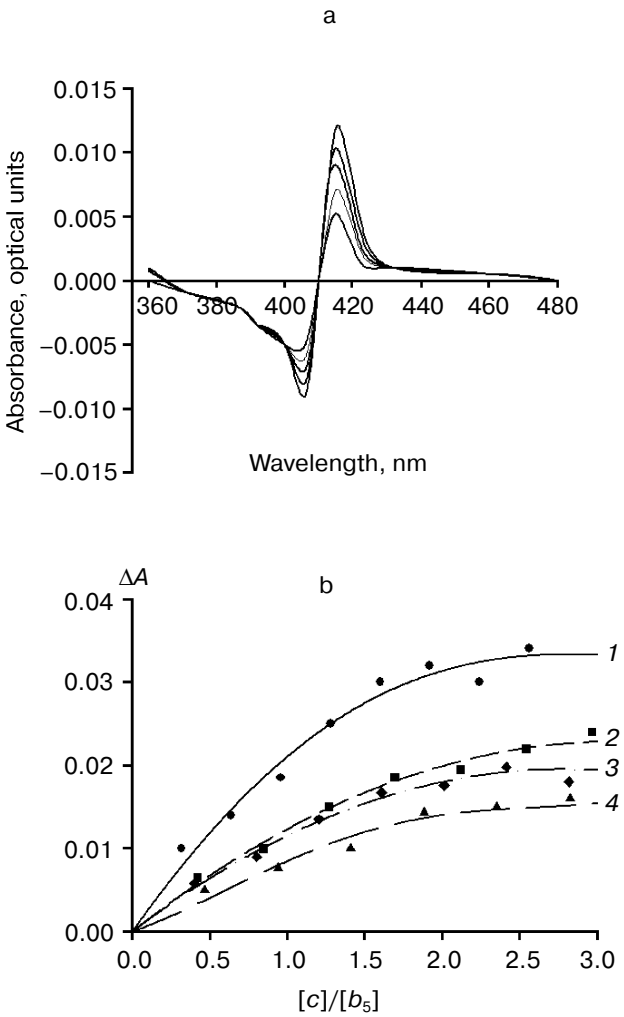



**Fig. 5.** Spectrophotometric titration of low molecular form of cytochrome $b_5$ with cytochrome *c*. a) Difference spectrum arising during interaction of cytochrome $b_5$ and cytochrome *c*; b) titration curves of wild-type cytochrome $b_5$ (*1*) and its mutants Glu42Lys (*2*), Asp65Ala (*3*), and Glu48Lys (*4*) with cytochrome *c*. The concentration of cytochrome $b_5$ during the titration was ~2.0 μM, while the concentration of cytochrome *c* was varied in the range 0.4-12.0 μM (2.5 mM potassium phosphate buffer, pH 7.4, 25°C).

hydrophobic interactions are involved in stabilization of the complex. There were no large differences in the character of interaction of immobilized cytochrome P450scc with full-length cytochrome $b_5$ and its mutant forms Glu48Lys, Asp65Ala, and Glu42Lys. Cytochrome $b_5$ and its mutants were eluted from the column by sodium chloride and sodium cholate in the concentration range 0.6-0.8 M NaCl and 0.25% sodium cholate (data not shown). However, the truncated form of either cytochrome $b_5$ or its mutant forms Glu48Lys, Asp65Ala, and Glu42Lys showed practically no binding with the column; they eluted in the void volume, indicating a critical role of the hydrophobic C-terminal sequence of cytochrome $b_5$ for its interaction with cytochrome P450scc and the important role of hydrophobic interactions in complex formation.

**Effect of recombinant cytochrome $b_5$ and its mutant forms on cholesterol side-chain cleavage activity of cytochrome P450scc.** As we showed earlier, cytochrome $b_5$ exerts a stimulating effect on the cholesterol side-chain cleavage reaction as catalyzed by cytochrome P450scc although the mechanism of this effect is unknown [22, 23]. The dependence of cholesterol side-chain cleavage activity of cytochrome P450scc on the concentration of cytochrome $b_5$ in the reconstituted system is shown in Fig. 6. At cytochrome P450scc/cytochrome $b_5$ ratio ~1 : 2 in the incubation mixture, there is an almost 100% increase in pregnenolone formation in the case of the wild-type cytochrome $b_5$ and its Asp65Ala and Glu48Lys mutants. The stimulating effect shown by the Glu42Lys mutant of cytochrome $b_5$ is only 40% of that seen with the wild-type heme protein, while the truncated forms of cytochrome $b_5$ and all the mutant forms were unable to affect the catalytic activity of cytochrome P450scc. These data indicate that the effect of cytochrome $b_5$ on the cytochrome P450scc catalyzed reaction requires that cytochrome $b_5$ have the hydrophobic C-terminal sequence and the negative charge at position 42 (Glu42). Residues Glu48 and Asp65 are not critical for the interaction of cytochrome $b_5$ with cytochrome P450scc. These data are in agreement with results of affinity chromatography of cytochrome $b_5$ on immobilized cytochrome P450scc showing that there was no large difference in the interaction of the two heme proteins after modification of Glu42, Glu48, and Asp65 of cytochrome $b_5$.

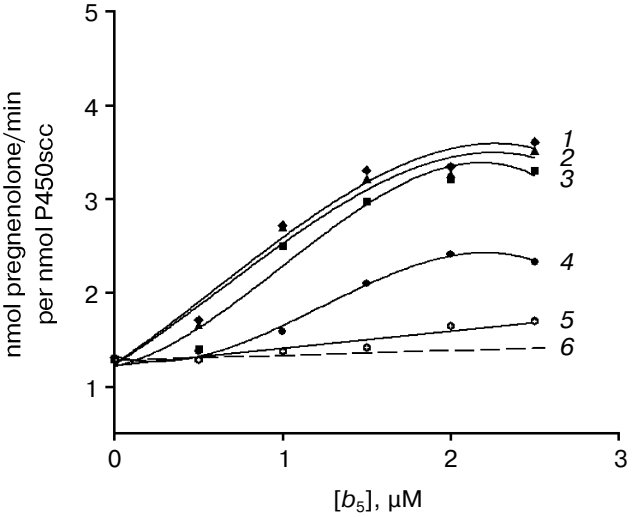



**Fig. 6.** Effect of cytochrome $b_5$ and its mutants on the activity of cytochrome P450scc in a reconstituted system (50 mM potassium phosphate buffer, pH 7.4, 0.05% Tween 20, 25 μM cholesterol, and deoxycorticosterone as internal standard). *1-3*) Activity in the presence of wild-type cytochrome $b_5$ and mutants Glu48Lys and Asp65Ala; *4*) Glu42Lys; *5*, *6*) in the presence of low molecular weight cytochrome $b_5$ and in the absence of cytochrome $b_5$. The concentrations of cytochrome P450scc, adrenodoxin, and adrenodoxin-reductase in the reconstituted system were 0.5, 1.0, and 0.5 μM, respectively. Cytochrome $b_5$ was added at concentrations in the range 0.25-3.0 μM.

**Effect of cytochrome $b_5$ and its mutant forms on 17,20-lyase activity of cytochrome P45017α.** Cytochrome $b_5$ plays an important role in the regulation of activity of cytochrome P45017α, one of the key enzymes in the biosynthesis of glucocorticoids and sex hormones [25, 26]. Thus, it was of interest to elucidate the effect of modification of residues Glu42, Glu48, and Asp65 of cytochrome $b_5$ on its ability to stimulate the 17,20-lyase activity of cytochrome P45017α in the oxidation of 17α-hydroxypregnenolone to dehydroepiandrosterone. As follows from Fig. 7, the mutant forms of cytochrome $b_5$, as compared to wild-type protein, are much less effective in stimulation of the 17,20-lyase activity of cytochrome P45017α. Thus, replacement of Glu42 and especially Glu48 by lysine results in a fourfold decrease in the stimulating ability of cytochrome $b_5$ on the 17,20-lyase activity of cytochrome P45017α compared to wild-type heme protein. However, the ability of mutant Asp65Ala of cytochrome $b_5$ to stimulate 17,20-lyase activity of cytochrome P45017α is decreased only 1.3-fold. The truncated forms of either wild-type cytochrome $b_5$ or its mutant forms in the incubation mixture has practically no affect on the activity of cytochrome P45017α, indicating that the stimulating effect on the 17,20-lyase activity of cytochrome P45017α by cytochrome $b_5$ requires the hydrophobic C-terminal sequence as well as the negative charges at Glu42 and Glu48.

These data indicate that ability of cytochrome $b_5$ to stimulate the 17,20-lyase activity of cytochrome P45017α to a significant degree is determined by the state of the amino acids in the "acidic" cluster of cytochrome $b_5$. This suggests that the effect of cytochrome $b_5$ on the 17,20-lyase activity of cytochrome P45017α requires the formation of a specific complex in which electrostatic interactions of the negatively charged residues Glu42 and Glu48 of cytochrome $b_5$ with the positively charged residues of cytochrome P45017α occur.

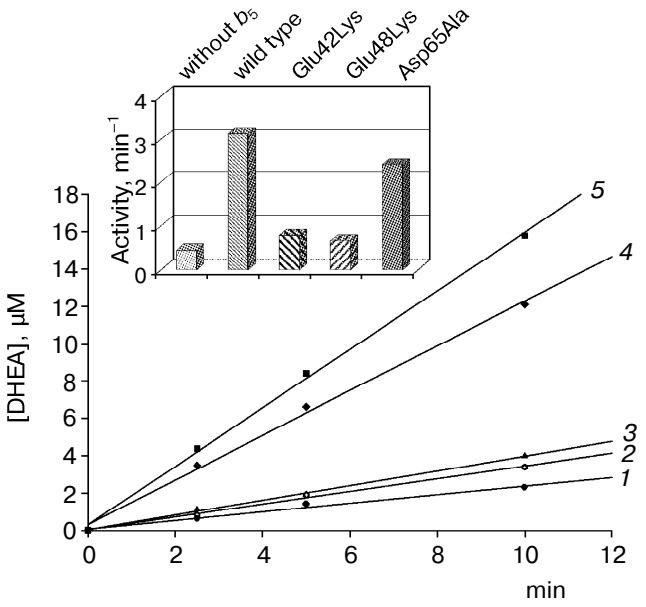



**Fig. 7.** Effect of cytochrome $b_5$ and its mutants on 17,20-lyase activity of cytochrome P45017α: *1*) in the absence of cytochrome $b_5$; *2*) in the presence of cytochrome $b_5$ mutant Glu48Lys; *3*) Glu42Lys; *4*) Asp65Ala; *5*) wild-type cytochrome $b_5$. The reaction mixture contained 0.5 μM bovine cytochrome P45017α, 1 μM recombinant rat NADPH-cytochrome P450 reductase, 0.5 μM recombinant cytochrome $b_5$, and 50 μM 17α-hydroxypregnenolone (50 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 37°C).

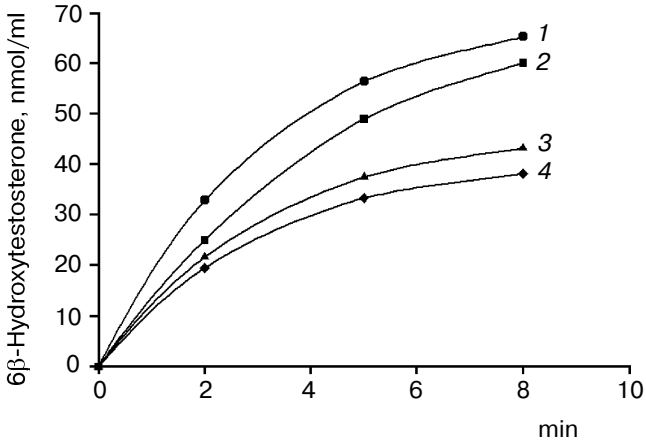



**Fig. 8.** Testosterone 6β-hydroxylase activity of recombinant cytochrome P4503A4 in the presence of cytochrome $b_5$ and its mutant forms: *1*) in the presence of wild-type cytochrome $b_5$; *2*) Asp65Ala; *3*) Glu42Lys; *4*) Glu48Lys. The reaction mixture contained 0.5 μM recombinant cytochrome P4503A4, 1 μM recombinant rat NADPH-cytochrome P450 reductase, 10 mM $MgCl_2$, 0.25 mg/ml CHAPS, 0.15 mg/ml DOPC, 100 μM testosterone, about 600,000 cpm [$^{14}$C]testosterone, 8 mM sodium isocitrate, and 0.1 IU/ml isocitrate dehydrogenase. The activity values were 32.8, 25, 21.6, and 19.4 min$^{-1}$ for wild-type cytochrome $b_5$ and its mutant Asp65Ala, Glu42Lys, Glu48Lys, respectively.

**Effect of cytochrome $b_5$ and its mutant forms on testosterone 6β-hydroxylase activity of recombinant cytochrome P4503A4.** The catalytic activity of cytochrome P4503A4 and, in part, 6β-hydroxylation of testosterone, is significantly stimulated by the addition of cytochrome $b_5$ to the incubation mixture, as shown both by experiments in reconstituted systems [7, 8, 46] and during simultaneous expression of cytochrome P4503A4 and cytochrome $b_5$ in *E. coli* [47, 48]. Data on the effects of different variants of cytochrome $b_5$ on testosterone 6β-hydroxylase activity of recombinant cytochrome P4503A4 in the reconstituted system are shown in Fig. 8. The activities of cytochrome P4503A4 in the presence of different forms of cytochrome $b_5$ were 32.8, 25, 21.6, and 19.4 min$^{-1}$ for wild-type cytochrome $b_5$ and the Asp65Ala, Glu42Lys, and Glu48Lys mutants, respectively. As in the case of cytochrome P45017α, replacement of Asp65 of cytochrome $b_5$ does not dramatically affect the ability of the mutant of cytochrome $b_5$ to stimulate the testosterone 6β-hydroxylase activity of cytochrome P4503A4, suggesting that Asp65 of cytochrome $b_5$ is not directly involved in the interaction with cytochrome P4503A4. However, removal of the negative charge of glutamate residues Glu42 and Glu48 by their substitution with lysine dramatically decreased the ability of these mutant forms of cytochrome $b_5$ to stimulate the activity of cytochrome P4503A4, confirming their direct involvement in electrostatic interaction with cytochrome P4503A4. As in the case of cytochrome P45017α, the presence in the incubation mixture of the truncated forms of cytochrome $b_5$ or its mutants had practically no affect on the activity of cytochrome P4503A4. Thus, the effect of cytochrome $b_5$ on testosterone 6β-hydroxylase activity of cytochrome P4503A4 requires the hydrophobic C-terminal sequence as well as the negative charges of residues Glu42 and Glu48.

Thus, the data suggest that glutamate residues Glu42 and Glu48 of cytochrome $b_5$ are directly involved in its interaction with both cytochrome P45017α and cytochrome P4503A4, while these residues play much less role in the interaction of cytochrome $b_5$ with cytochrome P450scc.

## DISCUSSION

Cytochrome $b_5$ has long been one of the favorite objects for the study of the mechanism of interaction of electron-transfer proteins and electron transfer between heme proteins in specific protein complexes. According to current ideas, electrostatic interactions between negatively charged amino acid residues surrounding the heme of cytochrome $b_5$ and the propionic acid residue of the heme and positively charged groups of the electron-transfer partner play an important role in complex formation between cytochrome $b_5$ and its electron-transfer partner.

It is thought that the prosthetic groups of the interacting proteins in the complex become close enough to each other for direct electron transfer to occur [27, 28].

The adequacy of this model has received support in experiments on chemical modification and site-directed mutagenesis of cytochrome $b_5$ in studies of its interaction either with the model partner cytochrome *c* [30, 31] as well as with the physiological partner, NADH-cytochrome $b_5$ reductase [11, 12]. Recently, it became evident that some amino acid residues of the "acidic" cluster of cytochrome $b_5$ are indeed involved in the interaction of the electron donor and electron acceptor proteins [29].

In the present work we used site-directed mutagenesis of acidic amino acid residues of the heme pocket of cytochrome $b_5$ (Fig. 9). According to the latest experimental data obtained using site-directed mutagenesis, there are at least two different groups of negatively charged amino acid residues in the so-called "acidic" cluster of cytochrome $b_5$. The residues Glu48, Glu49, and Asp65 appear to be involved in interaction mostly with electron acceptor proteins, and in part with cytochrome *c* (Fig. 9); residues Glu42, Asp71, and Glu74 located on the other side of cytochrome $b_5$ are mainly involved in complex formation with the electron donor protein NADH-cytochrome $b_5$ reductase [12, 29]. However, the participation of cytochrome $b_5$ in numerous reactions of microsomal oxidation catalyzed by different forms of cytochrome P450 suggests a much more complicated mechanism of interaction between the two proteins in which, together with electrostatic interactions involving the catalytic hydrophilic domain of cytochrome $b_5$, an important role in the interaction is also played by hydrophobic interactions involving the C-terminal domain of cytochrome $b_5$ [16-23]. In most recently published papers concerning heterologous expression and site-directed mutagenesis of cytochrome $b_5$, cDNA coding only the hydrophilic domain of cytochrome $b_5$ was predominantly used [12, 29-31]. Thus, it was of interest to investigate the effect of substitution of the charge of some amino acid residues of the "acidic" cluster of full-length cytochrome $b_5$ on the character of its interaction with microsomal cytochromes P450—cytochrome P45017α and P4503A4—as well as with mitochondrial cytochrome P450scc.

We recently developed a heterologous system for efficient expression of full-length rat cytochrome $b_5$ in *E. coli* by using the pCW$^+$ori vector that provides preparative amounts of both full-length and proteolytically modified (C-terminal sequence truncated) forms of cytochrome $b_5$ [32]. The experiments on site-directed mutagenesis of cytochrome $b_5$ presented in this paper were intended to elucidate the functional role of the negatively charged amino acid residues Glu42, Glu48, and Asp65, which have been previously identified as possible sites of interaction between cytochrome $b_5$ and well-known electron transfer partners such as cytochrome *c* as well as with mitochondrial cytochrome P450scc and microsomal cytochromes P45017α and P4503A4.

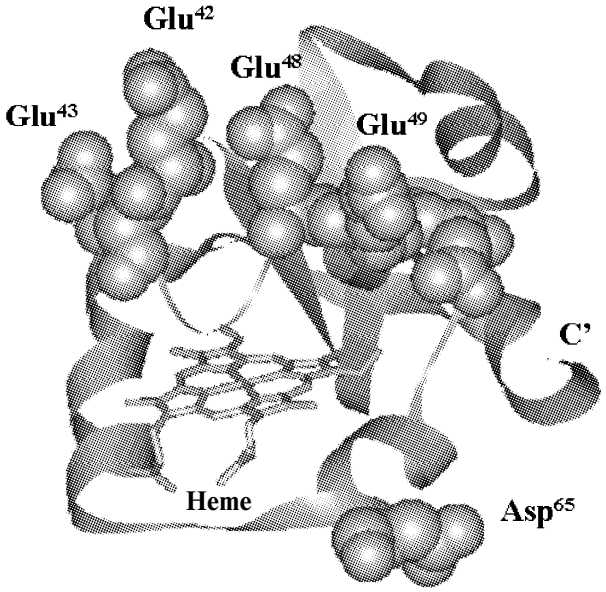



**Fig. 9.** Three-dimensional structure of rat cytochrome $b_5$ with indication of the negatively charged amino acid residues which are supposed to participate in interaction with electron transfer proteins. Coordinates for low molecular weight rat cytochrome $b_5$ were downloaded from the Protein Data Bank (PDB).

As follows from the data presented in the present paper, substitution of glutamate and aspartate residues at position 42, 48, and 65 (Glu42, Glu48, and Asp65), which are involved in formation of the so-called "acidic" cluster of cytochrome $b_5$, for lysine and alanine, respectively, results in significant disturbances in protein folding. A decrease in the content of α-helix compared to wild-type cytochrome $b_5$, as determined by circular dichroism data, was found in the Glu48Lys mutant (Fig. 4). It appears that after replacement of glutamate with an amino acid of opposite charge (lysine), the cytochrome $b_5$ molecule becomes less compact, and this is confirmed by the increased accessibility of the protein to endogenous proteolysis after the modification (table).

As follows from the data on modification of the N- and C-terminal fragments of the native cytochrome $b_5$ by cloning it into vectors pQE-32 and pCW$^+$ori HT to introduce additional histidine residues for application of metallo-affinity chromatography in preparative scale, it is more useful to use the six histidine cluster at the C-terminal sequence of cytochrome $b_5$ (vector pCW$^+$ori-$b_5$HT). Also, the insertion of a cysteine residue into cytochrome $b_5$ (which contains no cysteine in the native molecule) using construct pQE32-$b_5$ makes possible selective chemical modification of

cytochrome $b_5$ (e.g., to insert a fluorescent label) and also the use of thiopropyl-Sepharose for a purification procedure. It should be noted that, contrary to expectations, the approach using metallo-affinity chromatography to purify cytochrome $b_5$ proved not so efficient for purification of full-length forms of cytochrome $b_5$ mutants. Practically all expressed cytochrome $b_5$ is subjected to proteolytic modification despite the *E. coli* strain (JM109, DH-5α, and BL-21) and expression conditions used.

The experimental data obtained in the present work confirm the participation of modified amino acid residues in the interaction of cytochrome $b_5$ with cytochrome *c* [9, 10, 28]. The same amino acid residues appear to affect on the ability of cytochrome $b_5$ to stimulate the activity of microsomal cytochrome P45017α and P4503A4 in reactions of hydroxylation of 17α-hydroxypregnenolone and testosterone, respectively. However, as follows from experiments on immobilization of cytochrome $b_5$ done previously [32, 33] and on cytochrome P450scc done in the present work, as well as data on cholesterol side-chain cleavage activity of cytochrome P450scc in the presence of different forms of cytochrome $b_5$, an extremely important role in the stimulating effect of cytochrome $b_5$ is played by the hydrophobic part of cytochrome $b_5$, which is necessary both for interaction between two heme proteins and for stimulation of cytochrome P450-catalyzed reactions. The glutamate residue in position 42 of cytochrome $b_5$ (Glu42) appears to play an important role in this interaction, since, as shown in the present work, modification of this residue results in significant disturbance of the interaction of cytochrome $b_5$ with electron transfer partners and its ability to stimulate cytochrome P450-catalyzed reactions. However, the data of the present work suggest a different degree of participation of modified "acidic" amino acid residues of cytochrome $b_5$ in interactions with different types of cytochrome P450. Thus, the most dramatic effect on cytochrome P450scc-catalyzed reactions is caused by replacement of Glu42 for lysine, and less effect is caused by modification of Glu48. However, the Glu42 and Glu48 residues are of great importance for the effect of cytochrome $b_5$ on reactions catalyzed by microsomal cytochrome P4503A4 and P45017α. Residue Asp65 appear to be relatively unimportant for the interaction of cytochrome $b_5$ with different cytochromes P450 in comparison with its interaction with cytochrome *c*. This is in accordance with a conclusion made previously [29] on the specific nature of the interaction of cytochrome $b_5$ with different electron transfer partners.

Thus, the data of the present work indicate that, in contrast to the interaction of cytochrome $b_5$ with cytochrome *c*, complex formation between cytochrome $b_5$ and different cytochrome P450 isoforms requires, in addition to electrostatic interactions involving negatively charged amino acid residues surrounding the heme group of cytochrome $b_5$, also hydrophobic contacts involving the hydrophobic C-terminal part of cytochrome $b_5$.

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