ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 7, pp. 790-799. © Pleiades Publishing, Inc., 2006.
Published in Russian in Biokhimiya, 2006, Vol. 71, No. 7, pp. 972-983.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM06-014, April 16, 2006.

Expression of Outer Mitochondrial Membrane Cytochrome b_5 in *Escherichia coli*. Purification of the Recombinant Protein and Studies of Its Interaction with Electron-Transfer Partners

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Received January 24, 2006
Revision received February 23, 2006

Abstract—In the present work, we report expression in *Escherichia coli*, purification, and characterization of recombinant full-length cytochrome b_5 from outer mitochondrial membrane. Optimization of expression conditions for cytochrome b_5 from outer mitochondrial membrane allowed reaching expression level up to 10^4 nmol of the hemeprotein per liter of culture. Recombinant cytochrome b_5 from outer mitochondrial membrane was purified from cell lysate by using metal-affinity chromatography. It has physicochemical, spectral, and immunochemical properties similar to those of cytochrome b_5 from rat liver outer mitochondrial membrane. Immobilized recombinant mitochondrial cytochrome b_5 was used as affinity ligand to study its interaction with electron transfer proteins. By using this approach, it is shown that in interaction of NADPH:cytochrome P450 reductase with both forms of cytochrome b_5 an important role is played by hydrophobic interactions between proteins, although the contribution of these interactions in complex formation with NADPH:cytochrome P450 reductase is different for isoforms of cytochrome b_5.

DOI: 10.1134/S0006297906070121

Key words: microsomal cytochrome b_5, cytochrome b_5 from outer mitochondrial membrane, expression in *Escherichia coli*, affinity chromatography, purification

There are at least two different forms of cytochrome b_5 in hepatocytes [1]. One of these forms is bound to endoplasmic reticulum membranes (microsomal cytochrome b_5, b_5), while the other one with the outer mitochondrial membrane (mitochondrial cytochrome b_5, $b_5$om). Both forms of cytochrome b_5, being bound to the different structures of the cell, interact with membrane via its C-terminal, hydrophobic fragment, which is rather conservative among mammals, insects, plants, and prokaryotes [2, 3]. In contrast to microsomal, mitochondrial cytochrome b_5 is found mostly in mammals in liver and testis [4, 5]. Despite different localization in membrane structures of the cell and the fact that the two cytochromes b_5 are products of different genes, the hydrophilic fragments of both cytochromes b_5, containing heme [6], demonstrate a rather large degree of homology. Sequencing of genomes of some organisms confirmed the presence of cytochrome b_5 of outer mitochondrial membrane in some organs and tissues of higher organisms and allowed elucidation of their amino acid sequence, but did not clarify the functional role of this hemeprotein.

Microsomal and mitochondrial cytochrome b_5 have similar structural organization and include a hydrophilic heme-binding domain (approximately 100 amino acids) that retains spectral properties of hemeprotein, as well as a hydrophobic fragment responsible for binding with membrane, a domain containing about 40 amino acids. The later contains an inherent hydrophobic domain (approximately 20 amino acids) that is bound with membrane, and C-terminal hydrophilic fragment exposed on the surface of the membrane [7]. The C-terminal hydrophilic fragment carries information on the target of hemeprotein translocation in the cell after its biosynthesis—either to endoplasmic reticulum membrane or outer mitochondri-

Abbreviations: DTT) dithiothreitol; IPTG) isopropyl-β-thiogalactopyranoside; PMSF) phenylmethylsulfonyl fluoride.

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al membrane [8]. It is thought that the presence in the C-terminal hydrophilic fragment of mitochondrial cytochrome b_5 of positively charged amino acids determines its translocation to mitochondria. The removal of the positive charge results in translocation of the hemeprotein into endoplasmic reticulum membranes [8].

Alignment of the primary structures of microsomal and mitochondrial cytochrome b_5 (Fig. 1) indicates that the mitochondrial hemeprotein is slightly longer (146 amino acid residues against 134), contains an extra amino acid in the N-terminal sequence, and demonstrates significant differences in the C-terminal hydrophobic fragment [9]. Most interesting is the fact that the oxidation-reduction potential of mitochondrial cytochrome b_5 is more negative by 100 mV than the potential of microsomal cytochrome b_5 [10], and the heme is tightly bound to the apoprotein in the case of the mitochondrial cytochrome b_5 [11].

The main physiological role of microsomal cytochrome b_5 is participation in different types of electron-transfer reactions. Microsomal cytochrome b_5 transfers electrons from NADH to stearyl-CoA desaturase, which catalyzes an oxidation of fatty acids with formation of unsaturated lipids [12], participates in hydroxylation of cAMP-N-acetylneuraminic acid [13, 14], and serves as intrinsic electron transfer protein in NADH-dependent reduction of methemoglobin to form hemoglobin in erythrocytes [15]. The direct interaction of cytochrome b_5 with NADPH:cytochrome P450 reductase [16-18], different forms of cytochrome P450 [19-27], as well as with mitochondrial type cytochromes P450 (cytochrome P450cam [28, 29] and cytochrome P450scc [30, 31]) has been shown. Cytochrome b_5 plays a significant role in hydroxylation reactions of steroids catalyzed by cytochrome P45017α in endoplasmic reticulum membranes of some steroidogenic tissues [32, 33].

Cytochrome b_5 forms specific complexes with many electron transfer proteins such as cytochrome *c* and myoglobin [34-37]. Using chemical modification and modeling the presence of a specific domain of cytochrome b_5, which includes negatively charged residues Glu52, Glu48, and Asp64 and exposed heme propionate has been shown, and this domain is involved in electrostatic interactions with electron transfer proteins [38].

The functional role of mitochondrial cytochrome b_5 in monooxygenation reactions is unclear. There is evidence for participation of mitochondrial cytochrome b_5 in reduction of cytosolic semi-ascorbate via transport of electrons from NADH to NADH:cytochrome b_5 reductase and semidehydroascorbate reductase [39]. Recently it has been shown that mitochondrial cytochrome b_5 stimulates the reactions catalyzed by cytochrome P45017α during synthesis of sex hormones in testis [5, 40]. This finding has special interest since despite similarity of the tertiary structures of the soluble fragments of microsomal and mitochondrial cytochrome b_5 [8], they demonstrate completely different functional properties.

Recently, efficient systems for heterologous expression of microsomal cytochrome b_5 have been developed that can be used to purify recombinant cytochrome b_5 [41-48]. This approach has been successfully used to study structure–function relations in cytochrome b_5 by using site-directed mutagenesis [49, 50], elucidation of the mechanism of interaction of hydrophobic C-terminal sequence with membrane structures [51], as well as studies of the mechanism of electron transfer [52, 53]. However, there are no efficient methods for heterologous expression of the full-length mitochondrial cytochrome b_5 in bacteria, which significantly limits functional characterization of the mitochondrial isoform of cytochrome b_5.

The goal of the present work was to engineer expression vectors for heterologous expression of full-length

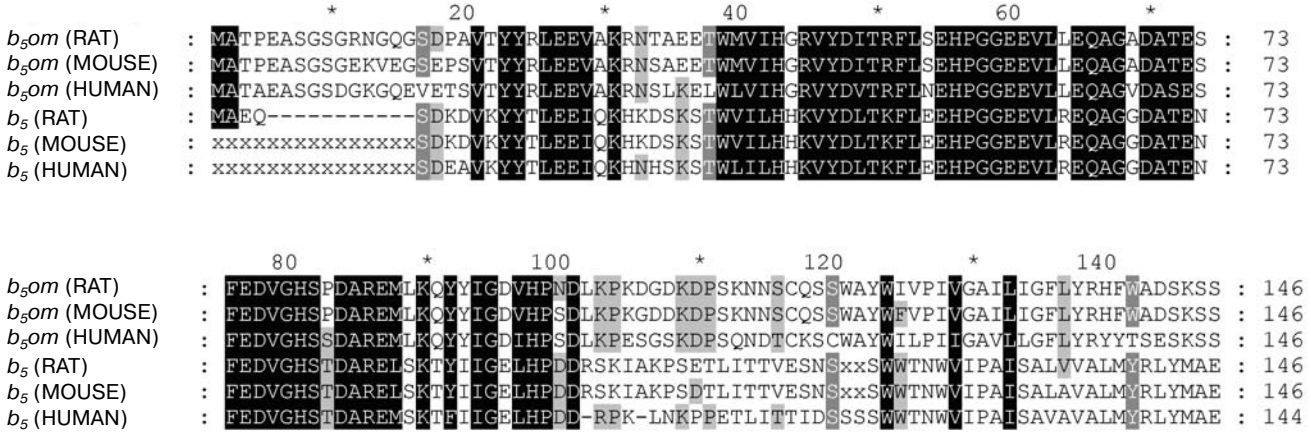

Fig. 1. Alignment of amino acid sequences of cytochrome b_5 ($b_5$om is outer mitochondrial membrane cytochrome b_5, b_5 is microsomal cytochrome b_5) from different species. The strictly conservative amino acid residues are shown in black and amino acid residues with 80 and 60% identity are indicated in dark- and light-gray, respectively.

mitochondrial cytochrome b_5 in *E. coli*, develop an efficient affinity purification procedure for recombinant cytochrome b_5, compare its physicochemical properties with that of microsomal cytochrome b_5, and apply the recombinant hemeprotein as an affinity ligand to study the interaction of immobilized full-length mitochondrial cytochrome b_5 with electron transfer proteins.

MATERIALS AND METHODS

Chemicals. In the present work we used cholesterol, pregnenolone, sodium cholate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), sodium dodecyl sulfate (SDS), trypsin, trypsin inhibitor, Coomassie R-250, CNBr-activated Sepharose 4B, Sephadex G-25, Sephadex G-75, and Sephacryl S-200 from Pharmacia (Sweden); albumin, NADPH, ω-aminobutyl-Sepharose 4B from Sigma (USA); Emulgen 911 and Emulgen 913 from Kao Atlas (Japan). Phenylmethylsulfonyl fluoride (PMSF), low-melting agarose, isopropyl-β-D-thiogalactopyranoside (IPTG), and dithiothreitol (DTT) were from BRL (USA). Bactotryptone and yeast extract were from Difco (USA).

Plasmids and bacterial strains. Chemical kits pCR^{TM}II (Invitrogene, USA) or pGEM-T vector (Promega, USA) were used to clone PCR reaction products. The expression vector pCWori$^+$ was kindly presented by Prof. M. R. Waterman (Vanderbilt University, USA).

Construction of expression plasmids. cDNA encoding full-length rat (*Rattus norvegicus*) mitochondrial cytochrome b_5 was amplified using polymerase chain reaction and the following primers:

OMB - 5′ Nde (TGTATCATATGGCTACTCCAGAAGCCAGCGGCAGCGGCAGG);

OMBch - 3′ Xba (GCATGTCGACTCTAGACGGAGGATTTGCTGTCAGCCCAG);

OMB - 3′ Sal (GCATGTCGACGAGGATTTGCTGTCAGCCCAG).

Primers were constructed based on the nucleotide sequence of rat mitochondrial cytochrome b_5 [54]. The 5′-primer (N-terminal primer) allows insertion into the N-terminal sequence of an additional *Nde*I restriction site, which was used later to clone the amplified sequence into the expression vector. The 3′- antisense primers (C-terminal primers) inset either *Xba*I or *Sal*I restriction sites. The first primer allows amplification of the cDNA encoding cytochrome b_5 without changes of the C-terminal sequence. The second primer introducing into the C-terminal sequence of cDNA of mitochondrial cytochrome b_5 an additional *Sal*I restriction site allows cloning this sequence into expression vectors containing His-tag cluster under the *Sal*I site. Insertion of the *Sal*I restriction site results in insertion of two additional codons encoding two additional amino acids before the His-tag—Ser-Thr-$(His)_6$COOH. The amplification products were ligated into pGEM-T vector (Promega). As a result we constructed two plasmids: pGEM-OMB(Xba) and pGEM-OMB-his(Sal). The correctness of manipulations was proved by sequencing of the resulting plasmids.

The *Nde*I-*Xba*I fragment of pGEM-OMB(Xba) vector was cloned into pCWori$^+$ vector, and the *Nde*I-*Sal*I fragment of pGEM-OMB-his(Sal) vector into pCWori$^+$HT vector. As a result, we constructed two types of expression plasmids: pCWori$^+$$b_5$om and pCWori$^+$$b_5$om-HT. The correctness of engineered plasmids was checked by restriction analysis and sequencing. Similarly, we cloned the insert coding mitochondrial cytochrome b_5 into pT7b_5 vector [46] under T7 promotor. As a result, we engineered pT7-$b_5$omHT plasmid coding full-length mitochondrial cytochrome b_5 with additional amino acids –Ser-Thr– and six histidine residues at the C-terminal sequence.

Expression of mitochondrial cytochrome b_5 in *E. coli*. Overnight cultures of *E. coli* DH5α using Taq promoter and BL21 using T7 promoter were diluted 1 : 500 with TBS-medium (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, 1 mM thiamine) containing 100 μg/ml of ampicillin and were incubated under shaking at 37°C until the absorbance at 600 nm (A_{600}) reached 0.3-0.4. After that, δ-aminolevulinic acid was added to final concentration 0.5 mM and the culture was incubated at 37°C until the absorbance at A_{600} reached 0.6-0.8. The expression of cytochrome b_5 was induced by addition of IPTG to final concentration 1 mM and culture carefully mixed at 20°C. After 45 min, the second portion of δ-aminolevulinic acid was added to final concentration 1.5 mM, and culture was incubated for 40-48 h at 20°C under shaking.

Purification of recombinant mitochondrial cytochrome b_5 from *E. coli*. To prepare spheroplasts, the cells were centrifuged at 5000*g* for 10 min at 4°C. The supernatant was removed. The pellet suspended in 15-20 ml of cold TES-buffer (100 mM Tris-acetate buffer, pH 7.6, containing 500 mM sucrose, 0.5 mM EDTA, 0.1 mM DTT) per g of wet weight of the cells. Lysozyme (solid) was added to the suspension to final concentration 0.5 mg/ml with subsequent drop addition of the same volume of 0.1 mM EDTA, pH 8.0, containing 0.1 mM DTT, and the suspension was mixed for 30 min at 4°C. Then the suspension was centrifuged at 5000*g* for 10 min. Spheroplasts were suspended in cold 50 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT (2 ml of buffer per g of wet cells), frozen at –70°C, and stored overnight. To prepare bacterial membranes, spheroplasts were thawed and

PMSF was added to final concentration 0.5 mM; the spheroplasts were sonicated using a UZDN-2T apparatus in regime of six 10-sec cycles with 30-sec intervals. To remove cell debris, the suspension was centrifuged at 5000*g* for 10 min. The supernatant was centrifuged at 100,000*g* for 60 min. The pelleted membranes were suspended in 50 mM sodium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.1 mM PMSF. Membranes obtained from bacterial strain DH5α, transformed with pCWori$^+$ plasmid, were washed with 100 mM sodium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM PMSF and suspended in the same buffer.

Membranes were solubilized on an ice bath by adding Emulgen 913 nonionic detergent to final concentration 1% (w/v) at detergent/protein ratio 3 : 1 (w/w) for 1 h. The purification procedure of recombinant mitochondrial cytochrome b_5 without His-tag did not significantly differ from the earlier described method [55]. After treatment with lysozyme, the cells were disrupted with ultrasonic treatment and membrane fraction was separated from cytosolic fraction of *E. coli* by ultracentrifugation at 100,000*g*. The concentration of cytochrome b_5 was determined from the absolute spectrum using molar extinction coefficient $\varepsilon_{413} = 117$ mM^{-1}·cm^{-1} or from the reduced/oxidized difference spectrum using molar extinction coefficient $\varepsilon_{424\text{-}409} = 185$ mM^{-1}·cm^{-1}, respectively [56].

The procedure of purification of mitochondrial cytochrome b_5 with His-tag at the C-terminal sequence consisted of metal-affinity chromatography on a column with Ni-NTA-Agarose (Qiagen, USA), the conditions of which were similar to that described earlier, but included an additional chromatographic step on DEAE-cellulose (DE-52; Whatman). The cytochrome b_5-containing fractions from the metal-affinity column were diluted 10-fold and applied to the column with DEAE-cellulose 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 by a gradient of 0.1-0.4 M NaCl in the same buffer and stored at −70°C.

Immobilization of recombinant mitochondrial cytochrome b_5. Cytochrome b_5 on CNBr-activated Sepharose 4B was immobilized according to the company protocol. The gel was equilibrated with 0.1 M sodium bicarbonate buffer, pH 8.3, and cytochrome b_5 was added. The suspension was mixed 1 h at room temperature and overnight at 0-4°C. This procedure results in immobilization of almost 90% of the cytochrome b_5 added. The resultant gel contained 100 nmol of cytochrome b_5 per ml of the gel. After blocking non-reacted groups with 0.1 M glycine, the gel was washed with 0.05 M phosphate buffer, pH 7.4, containing 1 M NaCl/0.3% sodium cholate.

Determination of the total heme in mitochondrial cytochrome b_5. The total heme in mitochondrial cytochrome b_5 was determined using the pyridine-hemechromogen method. Cytochrome b_5 (final concentration not less then 2 μM) was mixed with NaOH and pyridine to reach final concentrations 0.2 M and 20%, respectively. The samples were placed in two cells and sodium dithionite was added to the sample cell. The concentration of heme was calculated from the changes in absorbance at 557 and 575 nm using molar extinction coefficient $\varepsilon_{557\text{-}575} = 32.4$ mM^{-1}·cm^{-1}.

Immunochemical characterization of mitochondrial cytochrome b_5. Immunoblotting of mitochondrial cytochrome b_5 was carried out after electrophoresis under denaturing conditions [57], using polyclonal antibodies (rabbit) to rat microsomal cytochrome b_5 (dilution 1 : 300, 1 h). After washing to remove excess of antibodies, the nitrocellulose was treated with conjugate of peroxidase with goat antibodies against rabbit immunoglobulins (Amersham Co., USA, dilution 1 : 3000, 1 h). The nitrocellulose was stained with α-chloronaphthol (0.6 mg/ml) and 1 mM hydrogen peroxide.

Limited proteolysis of mitochondrial cytochrome b_5. To study the accessibility of mitochondrial cytochrome b_5 to proteolytic modification and degradation, the recombinant hemeprotein was treated with trypsin at protein/enzyme ratio 100 : 1 in 20 mM Tris-HCl buffer, pH 8.0, at 37°C during different time intervals.

Affinity chromatography of NADPH:cytochrome P450 reductase on cytochrome b_5-CNBr-Sepharose 4B. An aliquot of NADPH:cytochrome P450 reductase (10 nmol) was applied (10 ml/h) to the column (volume 2 ml), which was preliminarily equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 20% glycerol (buffer A). The column was washed with buffer A containing increasing concentration of NaCl: 0.1-1.0 M (three column volumes for each concentration).

To study the contribution of hydrophobic interactions in the interactions of cytochrome b_5 with NADPH:cytochrome P450 reductase, the column with immobilized proteins was equilibrated with buffer A containing 0.3 M NaCl and an aliquot of NADPH: cytochrome P450 reductase (10 nmol) containing 0.3 M NaCl was applied to the column. The column was washed with buffer A containing increasing concentrations of NaCl: 0.4-1.0 M (three column volumes for each NaCl concentration). Finally, the column was washed with buffer A containing 1.0 M NaCl and 0.3% sodium cholate.

Circular dichroism spectra. The circular dichroism spectra were recorded to determine the contribution and ratio of the elements of secondary structure of cytochrome b_5. The spectra were recorded using a JASCO J-20 spectropolarimeter (JASCO, Japan) under the following conditions: slit, 1 nm; response time, 2 sec; scan speed, 20 nm/min; temperature, 20°C. Each spectrum is a mean of five measurements. In the UV region (200-260 nm), the measurements were carried out in 0.1-cm optical path cells and the concentration of cytochrome b_5

was 20 μM in 20 mM Tris-HCl buffer, pH 7.4. To record spectra in the visible region of the spectrum (300-600 nm), cells with optical path 10 mm were used. Molar ellipticity was calculated using the following equation:

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

where θ is ellipticity (millidegree), C is protein concentration (M), and l is optical pathlength (cm).

The percentage of each element of secondary structure of cytochrome b_5 was calculated from UV circular dichroism spectra using the program CD Spectra Deconvolution 2.1.

Kinetics of reduction of cytochrome b_5 with NADPH:cytochrome P450-reductase. Reduction kinetics were measured in 50 mM Tris-HCl buffer, pH 7.4 (25°C) containing 100 μM NADPH and 4.3 μM cytochrome b_5. To remove oxygen from the reaction mixture, the solution without proteins was bubbled with argon for 5 min. The reaction was started by adding NADPH:cytochrome P450 reductase to final concentration 0.12 μM. The reaction was carried out under argon flow.

To elucidate the role of ionic and hydrophobic interactions in the interaction of NADPH:cytochrome P450-reductase and cytochrome b_5 during reduction, 0.3 M NaCl or 0.1% Triton X-100 were added to the reaction mixture. The kinetics of reduction of cytochrome b_5 was measured from the reduced minus oxidized difference spectrum of cytochrome b_5 at $A_{424\text{-}413}$ using molar extinction coefficient $\varepsilon_{424\text{-}413}$ = 185 $mM^{-1} \cdot cm^{-1}$. Spectra were measured using a Solar CM 2203 spectrophotometer (Solar, Belarus).

Analytical methods. The protein content of recombinant bacterial cells as well as purity of proteins was determined by SDS-PAGE in 12% gel according to Laemmli [57] using a Mini Protean II apparatus (Bio-Rad, USA). Recombinant protein was immunochemically identified using immunoblotting analysis. Chromatographic procedures were carried out using an apparatus including a Uvicord S, Microperpex peristaltic pump, and a 2210 two-channel recorder (LKB, Sweden).

The concentration of mitochondrial cytochrome b_5 was determined from the absolute spectrum of the purified oxidized mitochondrial cytochrome b_5 using molar extinction coefficient 117 $mM^{-1} \cdot cm^{-1}$ at 413 nm [58].

RESULTS AND DISCUSSION

Plasmid constructs used to express cytochrome b_5 from outer mitochondrial membrane. In the first step of the development of the system for heterologous expression of mitochondrial cytochrome b_5, we tried to use the approaches developed for expression of microsomal cytochrome b_5 in bacteria [58]. For that purpose, we constructed two vectors—pCWori$^+$b$_5$om encoding and expressing the wild type mitochondrial cytochrome b_5 and pCWori$^+$b$_5$omHT encoding and expressing cytochrome b_5 containing the additional sequence -Ser-Thr-6His before the transcription termination codon (Fig. 2). However, the preliminary experiments showed that recombinant bacteria *E. coli* DH5α, transformed with expression plasmid either without His-tag cluster pCWori$^+$b$_5$om or with it pCWori$^+$b$_5$omHT (Fig. 2), in contrast to microsomal cytochrome b_5, extremely inefficiently synthesize the mitochondrial cytochrome b_5. Nevertheless, by using metal-affinity chromatography, we were able to purify the recombinant protein and record its absolute spectra, but the expression level proved to be rather low and did not exceed 50 nmol of mitochondrial cytochrome b_5 per liter of culture.

Since the main difference between microsomal and mitochondrial forms of cytochrome b_5 is that the mitochondrial hemeprotein contains an additional N-terminal sequence (Fig. 1), we engineered several expression plasmids containing sequences coding forms of mitochondrial cytochrome b_5 truncated at the N-terminal sequence: pCWori$^+$b$_5$om2HT truncated by 11 amino acid residues; pCWori$^+$b$_5$om3HT truncated by 13 amino acid residues; and pCWori$^+$b$_5$om4HT truncated by 30 amino acid residues. However, modification of the N-terminal sequence of mitochondrial cytochrome b_5 did not significantly increase the expression level of the hemeprotein. Moreover, the removal of 30 amino acid residues results in inability of recombinant hemeprotein to correctly fold (data not shown).

The next step was changing of the strength of the promoter. Instead of *Taq* promoter that proved to be inefficient, we chose T7 promoter. The construct of the expression vector pT7b$_5$omHT is presented in Fig. 2. Transformation of BL-21 cells with pT7b$_5$omHT vector results in efficient expression of the mitochondrial cytochrome b_5 as indicated by the pink color of the bacteria. As in the case of microsomal cytochrome b_5, mitochondrial cytochrome b_5 is expressed in bacteria in the reduced state indicating that expressed recombinant cytochrome b_5 receives an electron from a bacterial partner having corresponding oxidation-reduction potential.

Optimization of the expression conditions (expression time, temperature, IPTG concentration) achieved an expression level close to 9000 nmol/liter of culture (12-15 g of wet cells), which appears to be the highest expression level reached for mitochondrial cytochrome b_5. Another interesting point of the developed system is that together with the holo-form of mitochondrial cytochrome b_5, the bacterial cells contain a significant amount of apo-form of the hemeprotein. Titration of the disrupted cells with exogenous heme results in its incorporation into apo-form with formation of holo-form of cytochrome b_5. Taking this into account, the yield of recombinant mitochondrial cytochrome b_5 reaches up to 12,000 nmol per liter of culture.

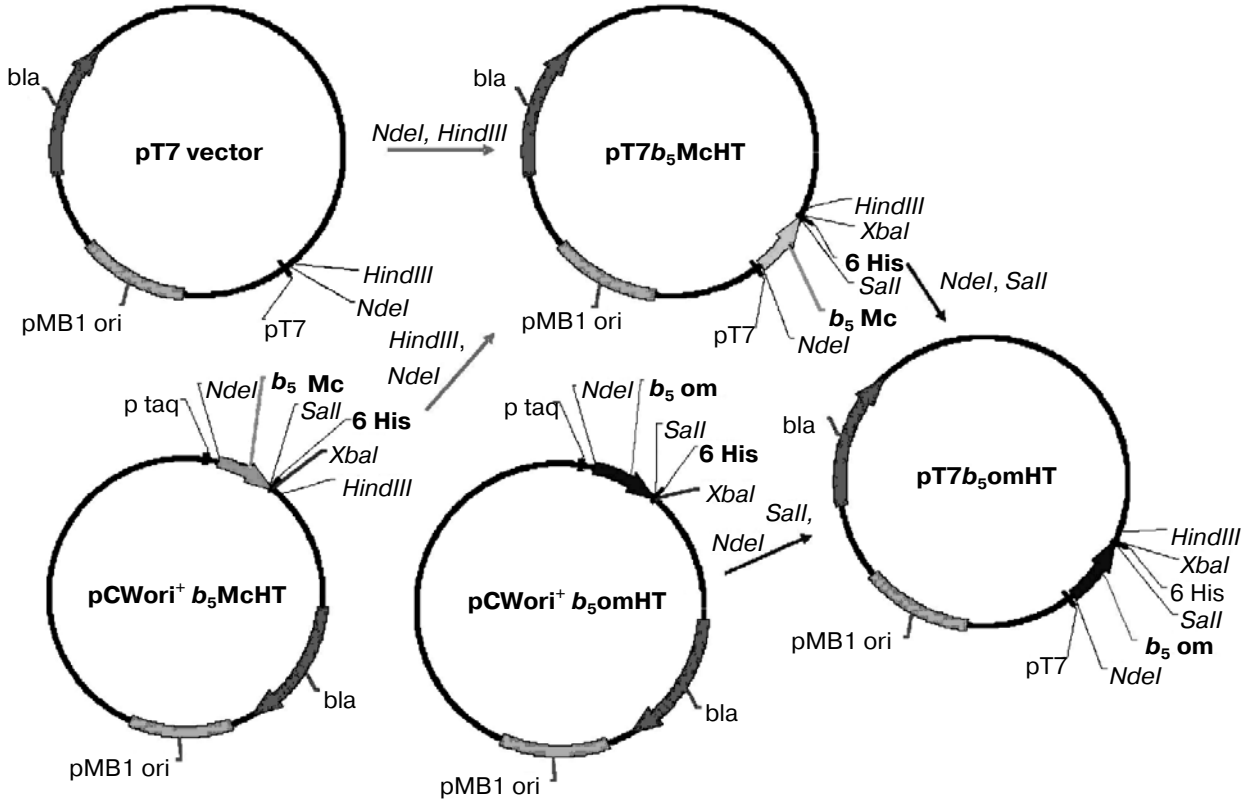

Fig. 2. Scheme of the construction of expression plasmid pT7b$_5$omHT containing cDNA encoding the full-length mitochondrial cytochrome b_5 under T7-promotor. In the first step, the fragment of pCWori$^+$b$_5$McHT plasmid containing cDNA for full-length microsomal cytochrome b_5 (*Nde*I-*Hind*III restriction fragment) was cloned into pT7 vector. The *Nde*I-*Sal*I fragment of this pT7b$_5$McHT vector was replaced with the *Nde*I-*Sal*I fragment of pCWori$^+$b$_5$omHT vector containing cDNA for full-length mitochondrial cytochrome b_5.

Metal-affinity chromatography of recombinant mitochondrial cytochrome b_5. The incorporation of additional histidine residues into the recombinant hemeprotein allows the use of metal-affinity chromatography for its purification, which significantly simplifies the purification procedure. After solubilization of membrane fraction the recombinant hemeprotein was applied to a Ni-NTA-agarose column (1 ml; Qiagen) preliminarily equilibrated with buffer A containing 0.2% sodium cholate and 5 mM imidazole. The column was washed with 10 column volumes of equilibrating buffer containing 0.5 M NaCl and 100 mM imidazole. The hemeprotein was eluted from the column with buffer A containing 0.2% sodium cholate, 0.5 M NaCl, and 400 mM imidazole. The presence of recombinant hemeprotein in fractions was detected based on absorbance at 413 nm characteristic for oxidized cytochrome b_5.

SDS-PAGE of the recombinant full-length mitochondrial cytochrome b_5 confirmed high purification degree (Fig. 3, inset) and indicated that the mitochondrial cytochrome b_5 has higher molecular weight than microsomal cytochrome b_5.

Spectral properties of recombinant mitochondrial cytochrome b_5. Figure 3 shows an absolute absorption

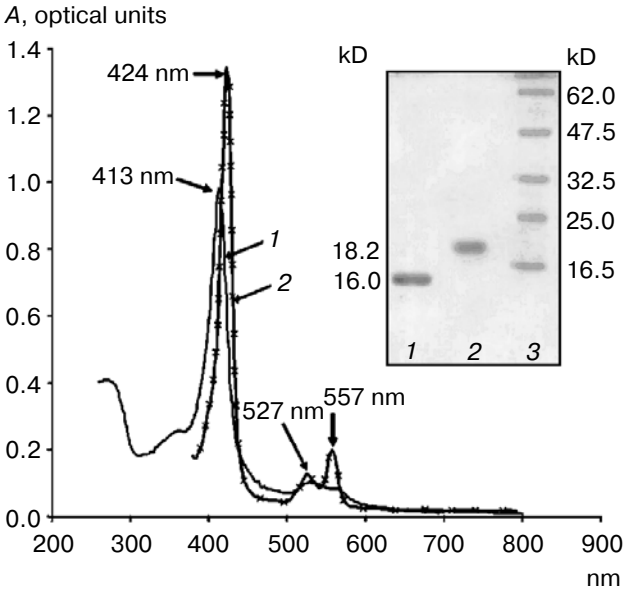

Fig. 3. Absolute absorption spectra of highly purified rat recombinant mitochondrial cytochrome b_5 (oxidized form (*1*), reduced form (*2*)). Inset: SDS-PAGE (15% polyacrylamide gel) of microsomal (*1*) and mitochondrial (*2*) cytochrome b_5; *3*) molecular weight markers.

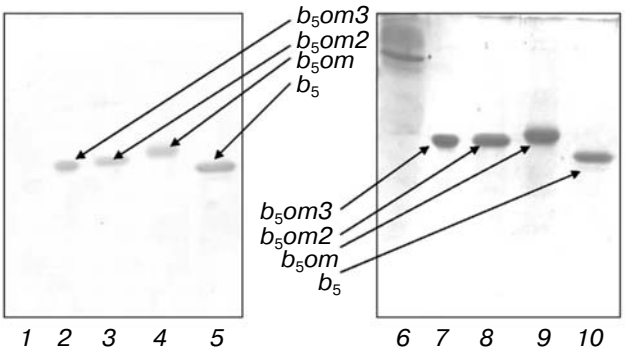

Fig. 4. Immunoblotting analysis of isoforms of cytochrome b_5 using polyclonal antibodies against microsomal cytochrome b_5 (*1-5*) and SDS-PAGE in 12% gel (*6-10*). Goat (*Capra hircus*) cytochrome P450-17α-hydroxylase was used as a negative control (*1*, *6*).

spectrum of highly purified full-length recombinant cytochrome b_5 from outer mitochondrial membrane. The oxidized absolute absorption spectrum of recombinant mitochondrial cytochrome b_5 is very similar to that of the microsomal hemeprotein [58] and has an absorption maximum in the Soret region at 413 nm and two small peaks in the longer wavelength region of the spectrum. The ratio of absorbance in the UV and Soret region being 2.8 confirms a high degree of purity of the recombinant mitochondrial cytochrome b_5. Reduction of mitochondrial cytochrome b_5 with sodium dithionite results in dramatic changes in the absorption spectrum. The maximum of absorbance in the Soret region is significantly increased and shifts to 423 nm, while in the longer wavelength region of the spectrum two new peaks at 527 and 557 nm appear, which is characteristic for native cytochrome b_5.

By using vector constructs with T7 promotor, we succeeded in expression and purification of truncated at N-terminal sequence forms of mitochondrial cytochrome b_5 ($b_5$om2 and $b_5$om3). The SDS-PAGE indicated high purity of the N-terminal truncated forms of mitochondrial cytochrome b_5 and confirmed the fact of truncation of the C-terminal sequence of the hemeprotein (Fig. 4). The spectral properties of truncated forms of mitochondrial cytochrome b_5 did not change from that of the full-length cytochrome b_5 (data not shown).

The results of studies of the conformation of microsomal and mitochondrial cytochrome b_5 using circular dichroism in the visible and ultraviolet regions are shown in Table 1, where also presented are the calculated percentage of the elements of the secondary structure for these hemeproteins compared with the same numbers calculated from the crystal structure of the truncated forms of the hemeproteins. Moreover, for comparison data for N-terminal truncated forms of mitochondrial cytochrome b_5 ($b_5$om2 and $b_5$om3) are also presented. As follows from the table, mitochondrial cytochrome b_5 has less content of α-helix as compared to microsomal cytochrome b_5, but it is more enriched in the content of β-sheets.

Immunochemical properties of mitochondrial cytochrome b_5. To understand the immunochemical similarity between forms of cytochrome b_5, we carried out immunoblotting analysis of mitochondrial cytochrome b_5 in the presence of antibodies against microsomal cytochrome b_5 (Fig. 4). As follows from Fig. 4, the mitochondrial cytochrome b_5 and its truncated forms ($b_5$om2 and $b_5$om3) are recognized by polyclonal antibodies against microsomal cytochrome b_5, indicating the presence of common antigenic determinants in the two forms of cytochrome b_5.

Limited trypsinolysis of isoforms of cytochrome b_5. To further study the conformational differences between the two forms of cytochrome b_5, we used limited proteolysis of the hemeproteins with trypsin. As follows from Fig. 5, the treatment of microsomal cytochrome b_5 with trypsin results in cleavage of the membrane-bound domain with formation of fragments with molecular weights 11 and 5 kD, which is in agreement with the model of membrane organization of this hemeprotein. However, mitochondrial cytochrome b_5 is cleaved at several sites to form at least five bands visible in 12% polyacrylamide gel. Based on the protein standards and program Gel-Pro Analyzer 3.1, we calculated that the molecular weights of the fragments *a*

Table 1. Ratio of secondary structure elements in two isoforms of cytochrome b_5, %

Elements of secondary structure	Mc b_5	Mc b_5(1AW3)*	$b_5$om	$b_5$om2	$b_5$om3	$b_5$om(1B5M)*
α-Helix	33.9	35.11	18.1	33.0	27.2	21.43
Antiparallel β-sheet	10.9		25.3	8.0	11.0	
Parallel β-sheet	5.6		5.4	9.1	10.0	
β-Turn	18.9	12.77	19.4	16.8	18.4	16.67
Random coil	30.8		33.0	33.4	34.1	

* Ratio of secondary structure elements in isoforms of cytochrome b_5 determined from the crystal structure of the hydrophilic domains.

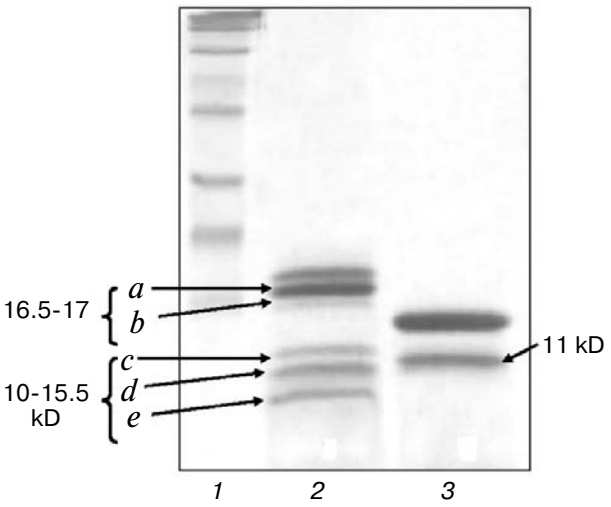

Fig. 5. Limited proteolysis of mitochondrial (*2*) and microsomal (*3*) cytochrome b_5. The bands *a*-*e* correspond to the fragments of mitochondrial cytochrome b_5 formed during limited proteolysis of the full-length hemeprotein. Microsomal cytochrome b_5 is cleaved by trypsin to form two main fragments: 11 and 5 kD (not shown). Lane *1* corresponds to protein standards (7-175 kD).

and *b* are in the range 16.5-17 kD, while the molecular weights of fragments *c*, *d*, *e* are in the range 10-15.5 kD.

Thus, the results of limited proteolysis clearly indicate significant differences in tertiary structure of microsomal and mitochondrial forms of cytochrome b_5.

Interaction of immobilized microsomal and mitochondrial cytochrome b_5 with electron transfer proteins. To directly study the specific interaction of mitochondrial cytochrome b_5 with electron transfer partners, we used an approach consisting of immobilization of cytochrome b_5 on a matrix and studying its interaction with soluble electron transfer partner. Under immobilization of mitochondrial cytochrome b_5 on CNBr-activated Sepharose 4B, about 90% of the hemeprotein was covalently linked with the matrix. The final gel contains about 100 nmol of mitochondrial cytochrome b_5 per ml of gel.

During studies of interaction of immobilized microsomal and mitochondrial cytochrome b_5 with NADPH:cytochrome P450 reductase, we found that at low ionic strength and in the absence of detergent neither form of cytochrome b_5 forms a tight complex with NADPH:cytochrome P450 reductase, which indicates an insignificant role of electrostatic interactions during complex formation between these two proteins (Fig. 6). However, at high ionic strength (0.3 M NaCl) and in the absence of detergent, microsomal cytochrome b_5 forms a tight complex with NADPH:cytochrome P450 reductase (at ionic strength up to 1 M NaCl). The dissociation of this complex occurs only when detergent is included in the buffer. This indicates that hydrophobic interactions play a predominant role in formation of the complex between microsomal cytochrome b_5 and NADPH:cytochrome P450 reductase (Fig. 6).

However, immobilized mitochondrial cytochrome b_5 did not form a complex with NADPH:cytochrome P450 reductase even in the presence of high ionic strength (Fig. 6).

Reduction of isoforms of cytochrome b_5 with NADPH:cytochrome P450-reductase. Reduction of the isoforms of cytochrome b_5 with NADPH:cytochrome P450 reductase was used to estimate the efficiency of electron transfer in different systems. As is evident from Table 2, in all studied cases microsomal cytochrome b_5 is reduced by NADPH:cytochrome P450 reductase several-fold more efficiently than mitochondrial hemeprotein. This appears to be connected with the difference in oxi-

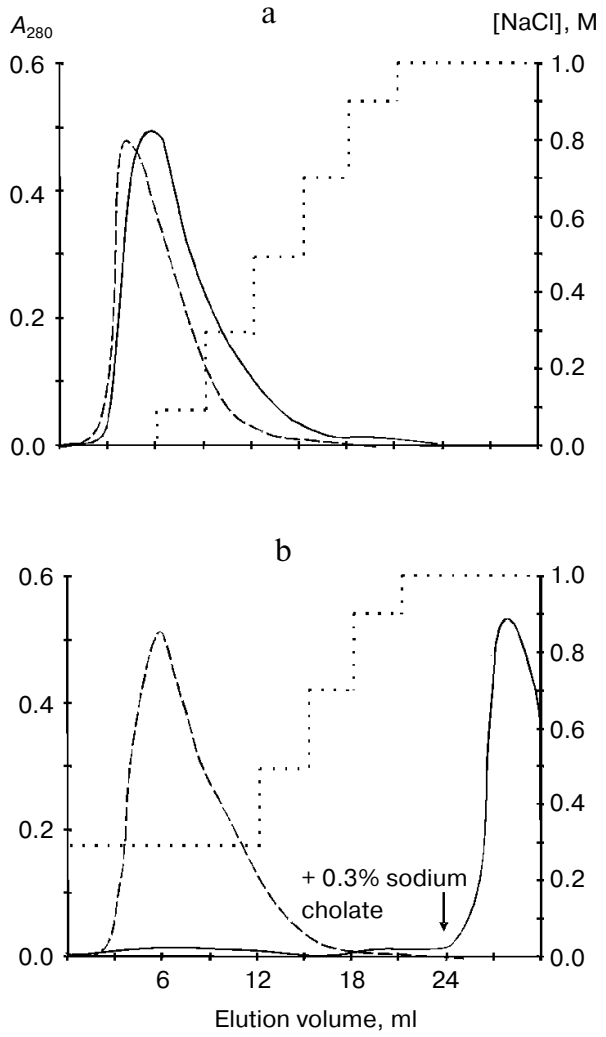

Fig. 6. Elution profile of the chromatography of NADPH:cytochrome P450 reductase on cytochrome b_5-Sepharose 4B (solid curves, microsomal cytochrome b_5; dashed curves, mitochondrial cytochrome b_5). NADPH:cytochrome P450 reductase was applied on the column in the absence of NaCl (a) or in buffer containing 0.3 M NaCl (b).

Table 2. Rate of reduction of isoforms of cytochrome b_5 with NADPH:cytochrome P450 reductase

Protein	Control, min^{-1}	% of control	Rate of reduction in the presence of 0.3 M NaCl, min^{-1}	% of control	Rate of reduction in the presence of 0.1% Triton X-100, min^{-1}	% of control
b_5	11.4	100	156.8	1380	4.5	40
$b_5$om	0.6	100	18.1	3000	1.35	225

dation-reduction potential between the isoforms of cytochrome b_5. Energetically it is easier for NADPH:cytochrome P450 reductase to transfer electrons to microsomal cytochrome b_5, the oxidation-reduction potential of which is equal 20 mV, than to mitochondrial isoform, whose oxidation-reduction potential is −100 mV.

Increasing ionic strength of the incubation mixture results in a 13-fold increase in reduction rate for microsomal cytochrome b_5 and 30-fold increase for mitochondrial cytochrome b_5. Both NADPH:cytochrome P450 reductase and cytochrome b_5 have net negative charge and interact via carboxyl groups with positively charged amino acid residues of cytochrome P450 [59]. Therefore, neutralization of the negative charges of cytochrome b_5 and NADPH:cytochrome P450 reductase by introducing NaCl into the system facilitates their interaction and consequently increases the reduction reaction rate. Neutralization of carboxyl groups of cytochrome b_5 and NADPH:cytochrome P450 reductase increases the rate of reduction of cytochrome b_5 [60]. Moreover, it is shown that a stimulating effect of Mg^{2+} on electron transfer from NADPH:cytochrome P450 reductase to cytochrome b_5 [61] is due to formation of salt bridges between negatively charged groups of the proteins and neutralization of the negative charges closely located on the protein surface.

It was recently shown [59] that the nonionic detergent Tergitol NP-10 (0.025% w/v) facilitates interaction of NADPH:cytochrome P450 reductase and cytochrome b_5, but the rate of reduction of cytochrome b_5 is decreased. This fact is explained taking into account a model according to which NADPH:cytochrome P450 reductase is surrounded by vesicles of detergent and interacts only with a small number of cytochrome b_5 molecules, also surrounded by detergent vesicles. Indeed, these molecules of cytochrome b_5 interact with NADPH:cytochrome P450 reductase and can reduce it. The other cytochrome b_5 molecules cannot interact with NADPH:cytochrome P450 reductase and, as an alternative, the reduced molecules of cytochrome b_5 from the nearest surroundings of NADPH:cytochrome P450 reductase can transfer electrons to oxidized cytochrome b_5 located outside NADPH-cytochrome P450 reductase. In any case, the reduction of cytochrome b_5 is taking place, but the rate of electron transfer from reduced to oxidized cytochrome b_5 is much less than the rate of direct reduction of cytochrome b_5 with NADPH:cytochrome P450 reductase. It is also necessary to stress that addition of detergent stimulates the reduction rate of mitochondrial cytochrome b_5, although the value of reaction rate is still 3.3-fold less than the rate of reduction of microsomal cytochrome b_5. This indicates different contribution of hydrophobic contacts to the interaction of NADPH-cytochrome P450 reductase with mitochondrial and microsomal cytochrome b_5.

Thus, in the present work we developed an efficient system for heterologous expression of full-length mitochondrial cytochrome b_5, an affinity method for purification of recombinant hemeprotein, and comparatively studied the physicochemical properties of microsomal and mitochondrial cytochrome b_5. The data presented indicate that despite structural similarity, the isoforms of cytochrome b_5 demonstrate different properties. In the interaction of NADPH:cytochrome P450 reductase and isoforms of cytochrome b_5, an important role is played by hydrophobic interactions, although the contribution of these interactions in complex formation with NADPH:cytochrome P450 reductase is different for the two forms of cytochrome b_5. The results obtained in the present work indicate different character of interaction of the two forms of cytochrome b_5 with NADPH:cytochrome P450 reductase. NADPH:cytochrome P450 reductase, being able to reduce both forms of cytochrome b_5, forms a specific complex with microsomal cytochrome b_5 mostly via hydrophobic interactions, while the role of hydrophobic interactions with mitochondrial cytochrome b_5 is negligible.

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