ORIGINAL ARTICLE

Preparation of apo-cytochrome b_5 utilizing heme transfer to apo-myoglobin

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Abstract

OBJECTIVES: Cytochrome b_5 (cyt b_5), a component of endoplasmic reticulum membrane, plays a role in modulation of activity of several cytochromes P450 (CYP). To elucidate the mechanism of such modulations it is necessary to evaluate not only the effect of native cyt b_5, but also that of apo-cyt b_5. To prepare apo-cyt b_5, heme transfer from native cyt b_5 to a protein with higher affinity toward the heme, the horse heart apo-myoglobin, was utilized.

METHODS: Butanone extraction was employed to prepare apo-myoglobin. Apo-cyt b_5 was separated from myoglobin by chromatography on DEAE-Sepharose. Mass spectrometry was utilized to characterize proteins eluted from DEAE- Sepharose.

RESULTS: The prepared apo-myoglobin was incubated with the cyt b_5 at pH 4.2 that is the optimal pH for heme transfer from cyt b_5 into apo-myoglobin. The apo-cyt b_5 protein was separated from myoglobin present in the reaction mixture by chromatography on a column of DEAE-Sepharose. Using such a procedure, 16% yield of apo-cyt b_5 that did not contain any heme in its molecule was obtained from the native rabbit cyt b_5. Oxidized and reduced forms of the apo-b_5 reconstituted with heme exhibit the same absorbance spectra as native cyt b_5. The prepared apo-cyt b_5 reconstituted with heme can receive electrons from NADPH:CYP reductase.

CONCLUSION: A biologically active apo-cyt b_5 was prepared using transfer of heme from cyt b_5 to horse heart apo-myoglobin by the procedure described here.

INTRODUCTION

Cytochrome b_5 (cyt b_5), a component of endoplasmic reticulum membrane, is a heme protein with molecular mass of 15 800 (rabbit cyt b_5). It is composed of two functional domains, a soluble heme-containing core, and a short hydrophobic C-terminal tail, which anchors the protein into the microsomal membrane (Schenkman & Jansson, 2003). Cyt b_5 has been shown to stimulate, inhibit or have no effect on reactions catalyzed by cytochromes P450 (CYP), the key enzymes involved in metabolism of xenobiotics such as drugs and toxic and carcinogenic compounds, as well as endogenous compounds such as prostaglandins, fatty acids and steroid hormones (Guengerich et al. 1976, Gonzalez & Gelboin, 1992, Ortiz de Montellano, 1995; Shenkman & Jansson, 1999, 2003,

Abbreviations:

BCA	– bicinchoninic acid
BSA	– bovine serum albumin
cm	– centimeter
°C	– centigrade grades
CYP	– cytochrome P450
cyt b_5	– cytochrome b_5
DEAE	– diethylaminoethyl-Sepharose
DLPC	– L-α-dilauroyl-sn-glycerol-3-phosphocholine
EDTA	– ethylene diamine tetraacetic acid
MeCN	– acetonitrile
min	– minute
M	– mol/liter
mM	– milimol/liter
mL	– mililiter
µL	– microliter
mg	– miligram
ng	– nanogram
nm	– nanometer
NADPH	– nicotinamide adenine dinucleotide phosphate (reduced)
RPM	– rotation per minute
UV/Vis	– ultraviolet/visible

Stiborova et al. 2006; Sulc et al. 2008). The effect of cyt b_5 is strongly dependent on individual CYP isoforms, their substrates and experimental conditions (Schenkman & Jansson, 2003). Several hypotheses trying to explain the influence of cyt b_5 on CYP reactions have been proposed. One of them suggests a role of cyt b_5 in a direct transfer of the second electron to the CYP enzyme, which is considered to be the rate limiting step in the catalytic cycle of the CYP monooxygenase reaction (Guengerich et al. 1976). The electron transfer from reduced cyt b_5 to CYP is faster than the input of electron from NADPH:CYP reductase (Hildebrandt & Estabrook, 1971; Schenkman & Jansson, 1999). Due to this stimulation effect, CYPs are prevented from uncoupling of partially activated oxygen. The release of superoxide and hydrogen peroxide appears to various extents in all monooxygenase reactions, but also depends both on a CYP isoform and a substrate. Moreover, the addition of cyt b_5 enhances the stability of the oxy-CYP complex, therefore higher amount of activated oxygen is available to form hydroxylated product (Schenkman & Jansson, 2003).

Another possible mechanism of the cyt b_5 action is the formation of a complex between cyt b_5 and CYP, which can receive two electrons from NADPH:CYP reductase in a single step, one for reduction of CYP and another for that of cyt b_5 (Schenkman & Jansson, 2003). While CYP without cyt b_5 has to undergo two separate interactions with NADPH:CYP reductase to complete one catalytic cycle, in the case of the presence of cyt b_5, only one single interaction of complex of CYP and cyt b_5 with NADPH:CYP reductase is sufficient; cyt b_5 provides the second electron to CYP promptly after oxygen binding. Interaction of cyt b_5 with CYP may also induce conformational changes in CYP proteins leading to breakdown of oxygenated hemoprotein complex with substrates to products. This hypothesis is based on findings showing that not only holoprotein of cyt b_5, but also its apo-form (devoid of heme), which is not capable of electron transfer, can contribute to stimulation effects (Yamazaki et al. 1996, 2001, 2002; Auchus et al. 1998, Kotrbova et al. 2009).

It is clear from such investigations that studies utilizing apo-cytochrome b_5 (apo-cyt b_5) are necessary to explain the mechanisms of cyt b_5 effects on CYP-catalyzed reactions. Preparation of this apo-protein in an appropriate quality is hence crucial for further studies in this field.

Several different approaches to prepare the apo-hemoproteins, including apo-cyt b_5, in a large scale were utilized. One of them, efficiently removing a heme part of this cytochrome and other hemoproteins, was the extraction of heme by acid acetone treatment (Rossi-Fanelli et al. 1958, Cinti & Ozols, 1975; Yamazaki et al. 1996; Mrazova et al. 2008). Nevertheless, during this procedure, a part of such apo-cyt b_5 protein preparations seemed to become denatured; irreversible precipitation of the apo-cyt b_5 protein was found (Mrazova et al. 2008). Therefore, other procedure, which gently removes the heme cofactor to prepare the pure apo-hemoproteins including apo-cyt b_5 without altering the native protein conformation, should be developed. One of them might be an expression of recombinant hemoproteins in the system without heme precursor - δ-aminolevulic acid (Miksanova et al. 2006; Kotrbova et al. 2009). Indeed, recently, we have used such a procedure, utilizing a heterologous expression of apo-b_5 in *E. coli*. In contrast to preparation of holoprotein of cyt b_5 by a heterologous expression (Mulrooney & Waskell, 2000), the precursor of heme biosynthesis, the δ-aminolevulinic acid, was not added to bacterial culture. We have demonstrated that such a procedure leads to expression of about 99% of cyt b_5 protein in its apo-form (Kotrbova et al. 2009). Residual heme in this apo-cyt b_5 preparation can be of a heme from *E. coli* used as an expression system, which can be incorporated into the produced apo-cyt b_5.

Another procedure suitable to prepare apo-cyt b_5 might utilize heme transfer from cyt b_5 to another heme protein in its apo-form that has the higher affinities to heme. The affinities of apo-hemoproteins for heme are very large, showing equilibrium dissociation constants in the 10^{-10}-10^{-15} M region (Hargrove et al. 1996; Miksanova et al. 2006). In the holoproteins, the heme prosthetic group appears to be stabilized by a large number of hydrophobic (van der Waals) and electrostatic contacts. The vinyl groups are pointing toward the protein interior and surrounded by non-polar aliphatic and aromatic side chains, whereas the propionates point toward the solvent and interact with a variety of charged or polar amino acids. These interactions and the extremely small dissociation constants imply a high degree of specificity in the binding process (Hargrove et al. 1994). However, a variety of experimental evidence suggests that the association of heme with apo-globin is little affected by globin structure (Hargrove et al. 1996).

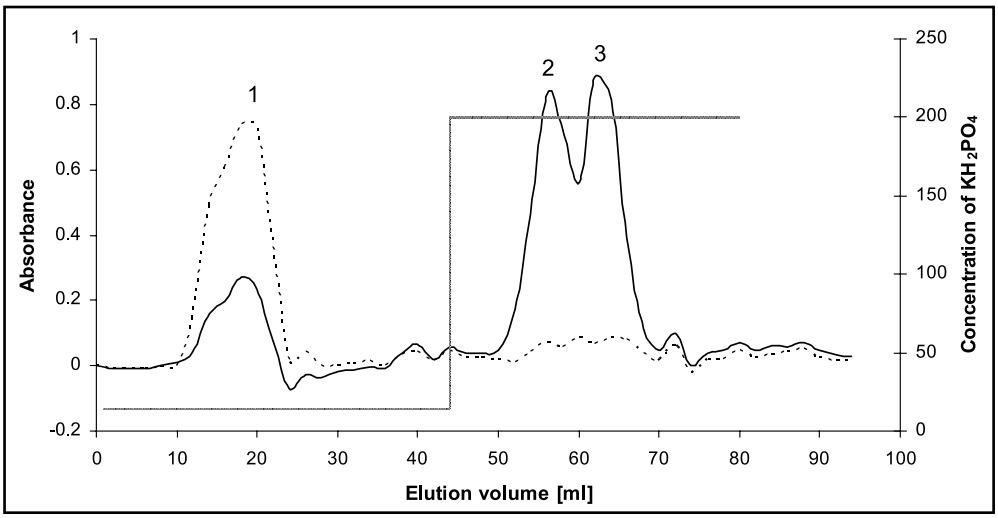

Figure 1: Chromatography of proteins of incubation mixture used for preparation of apo-cyt b_5 on DEAE-Sepharose CL6B. Peak 1 - myoglobin; peak 2 - complex of cyt b_5 and myoglobin; peak 3 - apo-cyt b_5. Absorbance at 280 nm is shown as *solid line*, and at 409 nm as *dash line*. Concentration of KH_2PO_4 (*dotted line*). Experimental conditions are described in Material and methods.

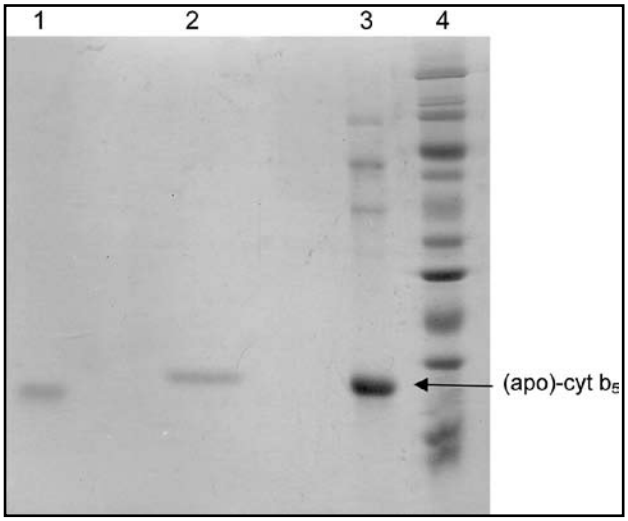

Figure 2: SDS-PAGE (15% separating gel, staining with Coomassie Blue) of apo-cyt b_5 and protein peaks 2 and 3 obtained by chromatography on DEAE-Sepahrose. Lanes 1, protein peak 2 (complex of myoglobin and cyt b_5), lane 2, protein peak 3 (final apo-cyt b_5 preparation), lane 3, native rabbit hepatic cyt b_5, lane 4, molecular weight standard (Sigma wide range).

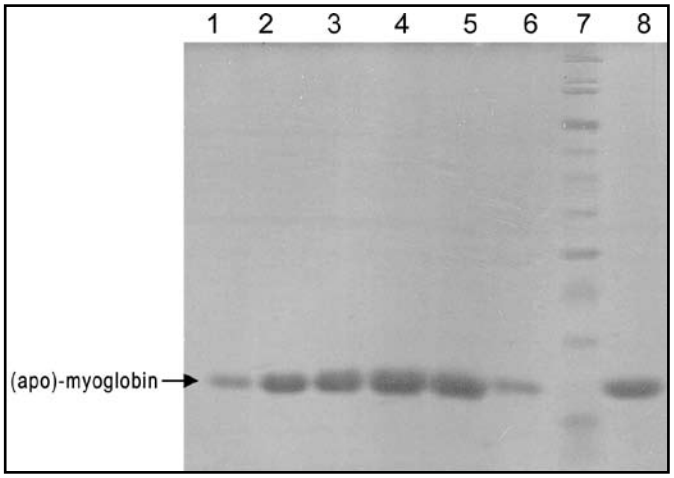

Figure 3: SDS-PAGE (15% separating gel, staining with Coomassie Blue) of horse heart myoglobin and protein peak 1 obtained by chromatography on DEAE-Sepahrose. Lane 1 – 5, protein fractions eluted from DEAE-Sepharose with 12 – 20 ml of 15 mM KH_2PO_4, containing 0.1 mM EDTA, 0.1% sodium cholate, 20% glycerol, pH 6, lane 6 - horse heart myoglobin standard, lane 7 - molecular weight standard (Sigma wide range), lane 8 – pooled protein peak 1 obtained by chromatography on DEAE-Sepharose.

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gi|7546624, Chain A, Myoglobin (Horse Heart) Wild-Type
     1 GLSDGEWQQV LNVWGKVEAD IAGHGQEVLI RLFTGHPETL EKFDKFKHLK
    51 TEAEMKASED LKKHGTVVLT ALGGILKKKG HHEAELKPLA QSHATKHKIP
   101 IKYLEFISDA IIHVLHSKHP GDFGADAQGA MTKALELFRN DIAAKYKELG
   151 FQG

gi|159162212, Chain A, Solution Structure Of Oxidized Microsomal Rabbit
Cytochrome B5
     1 DKDVKYYTLE EIKKHNHSKS TWLILHHKVY DLTKFLEEHP GGEEVLREQA
    51 GGDATENFED VGHSTDAREL SKTFIIGELH PDDRSKLSKP METL

gi|117811, Cytochrome b5
     1 MAAQSDKDVK YYTLEEIKKH NHSKSTWLIL HHKVYDLTKF LEEHPGGEEV
    51 LREQAGGDAT ENFEDVGHST DARELSKTFI IGELHPDDRS KLSKPMETLI
   101 TTVDSNSSWW TNWVIPAISA LIVALMYRLY MADD
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Figure 4: Protein sequences of horse heart myoglobin and rabbit cytochrome b_5. Peptides identified by LIFT MALDI-TOF MS/MS (see Table 2) are in red.

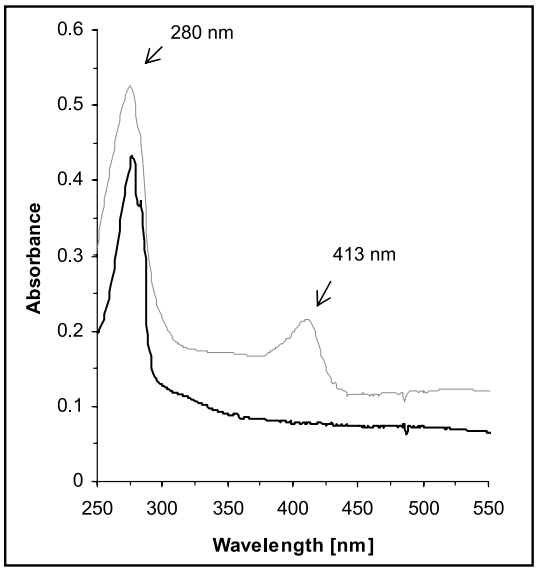

Figure 5. Absorbance spectra of the purified apo-cyt b_5 preparation *(solid line)* and the same protein reconstituted with heme *(dash line)*.

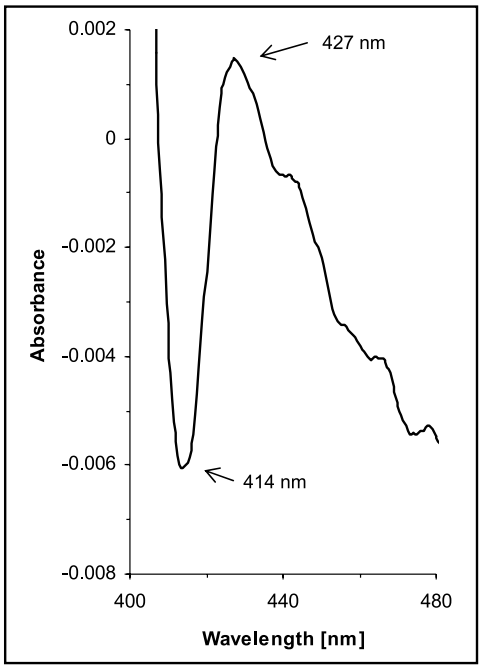

Figure 7. Difference spectrum (reduced minus oxidized) of purified apo-cyt b_5 reconstituted with heme

Here, we developed a novel procedure to prepare apo-cyt b_5, utilizing a protein with higher affinity toward the heme, the apo-myoglobin from horse heart. A biologically active apo-cyt b_5 was prepared using transfer of heme from cyt b_5 to this apo-protein.

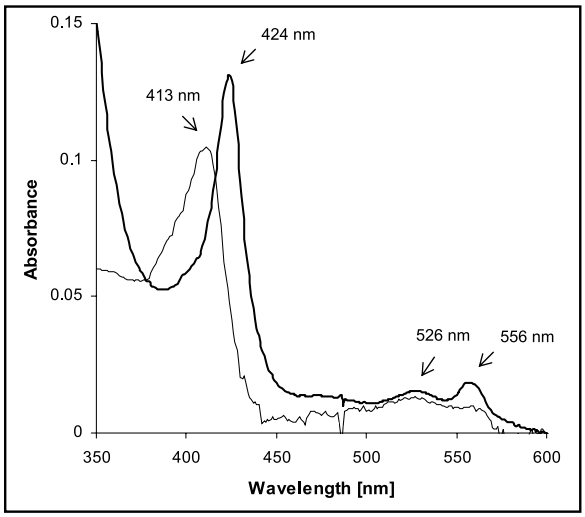

Figure 6. Absorbance spectra of purified apo-cyt b_5 reconstituted with heme. Oxidized form is represented by *dashed line* and sodium dithionite-reduced form by *solid line*.

Table 1. MALDI-TOF mass spectra UltraFLEX of differentiated signals of protein peaks eluted obtained by chromatography on DEAE-Sepharose

Peak 1 m/z	Peak 2 m/z	Peak 3 m/z
748.43	950.5	2279.2
1271.7	2279.2	
1606.8		

MATERIAL AND METHODS

Chemicals and enzymes: L-α-dilauroyl-sn-glycerol-3-phosphocholine (DLPC), nicotinamide adenine dinucleotide phosphate (reduced) (NADPH), myoglobin and sodium cholate were obtained from Sigma-Aldrich (USA). Sodium dodecyl sulfate (SDS) was from Fluka (Switzerland) and DEAE-Sepharose CL6B from GE Healthcare Bio-Sciences AB (Sweden). Rabbit liver cyt b_5 was isolated in our laboratory from rabbit liver microsomes by the procedure described by Roos (1996). NADPH:CYP reductase was purified from rabbit liver as described previously (Dignam & Strobel, 1977).

Preparation of apo-myoglobin from horse heart myoglobin: In the first step, a heme moiety was extracted from the native horse heart myoglobin by butanone extraction by the slightly modified method of Rossi-Fanelli et al. (1958), optimized in our previous work for preparation of apo-myoglobin from the equine skeletal muscle (Mrazova et al. 2008). pH of the horse heart myoglobin solution (100 mg of the horse heart myoglobin in 40 ml distilled water) was adjusted to pH 2.5 by 1 M HCl. The solution was moved to the separation funnel and equal volume of 2-butanone was added.

Table 2. Results of LIFT MALDI-TOF MS/MS spectra

m/z	Sequence	Protein name	Protein number	MW	Mowse Score	Identity score
748.43	K.ALELFR.N	Chain A, myoglobin (horse heart) wild-type	gi/7546624	16941	55	> 37
1271.7	R.LFTGHPETLEK.F				71	> 38
1606.8	K.VEADIAGHGQEVLIR.L				168	> 38
950.5	F.RNDIAAKY.K	Chain A, myoglobin (horse heart) wild-type	gi/7546624	16941	55	> 40
2279.2	F.IIGELHPDDRSKLSKPMETL.-	Chain A, Solution structure of oxidized microsomal rabbit cyt b_5	gi/159162212	10854	63	> 60

Such a mixture was slowly shaken 3 times for 5 minutes and than placed for 10 minutes into the cold room (8 °C). Aqueous phase was dialyzed at 8 °C against 2 liters of distilled water for 24 hours and than against 2 liters of 10 mM Tris, pH 8.0 for 2 days. The solution was concentrated in an Amicon stirred cell using a PM-10 membrane (Millipore). In order to prevent the precipitation of the apo-myoglobin, pH was adjusted to 5 using 2 M acetic acid before it was stored at -80°C (Mrazova et al. 2008).

Protein determination. Protein concentrations were determined by the bicinchoninic acid protein assay (Wiechelman et al. 1988) using bovine serum albumin as a standard.

Preparation of apo-cyt b_5 by heme transfer to apo-myoglobin. 1.3 mg of purified rabbit cyt b_5 dissolved in 1 mL of 10 mM KH_2PO_4, pH 6 (Buffer A) was mixed with 2.6 mg of horse heart apo-myoglobin dissolved in 0.72 mL of Buffer A (the molar ratio of proteins was 1:2) and than the mixture was diluted to approximately 10 mL with Buffer A. This mixture was adjusted to pH 4.2 using 1 M HCl (based on previous study showing this pH to be optimal for heme transfer) (Mrazova et al. 2008) and incubated for 10 minutes at ambient temperature. The heme transfer was observed as a shift of absorption maximum of heme from 413 to 409 nm during 10 minutes, measured using a Hewlett Packard 8453 UV spectrophotometer. Afterwards a small amount of 0.5 M NaOH was added to the mixture to obtain pH of 6.0. The incubation mixture was then applied onto a column of DEAE-Sepharose, where components of the incubation mixture were separated.

Chromatography on DEAE-Sepharose: All procedures were performed at 4 °C. A column of DEAE-Sepharose (1.5 x 10 cm) was equilibrated with 0.5 liter of 15 mM KH_2PO_4, containing 0.1 mM EDTA, 0.1% sodium cholate, 20% glycerol, pH 6 (Buffer B). The incubation mixture, which was used for preparation of apo-cyt b_5 was applied onto a column of DEAE-Sepharose at a flow rate of 1 mL/min and the column was washed with approximately 45 mL of Buffer B until myoglobin was eluted and the absorbance of the eluent at 280 nm decreased to zero (***Figure 1***). Then a step elution of further proteins of the incubation mixture, using 200 mM KH_2PO_4 containing 200 mM KCl, 0.1% sodium cholate, 20% glycerol, pH 6 (Buffer C), was performed (Figure 1). Protein fractions separated with chromatography on a column of DEAE-Sepharose were monitored at 280, 409 and 412 nm (Hewlett Packard 8453 UV spectrophotometer) and analyzed with SDS-PAGE (Laemmli, 1970), using 15% separating gel and stained with Coomassie Brilliant Blue R-250 (Krizkova et al. 2008). Fractions containing proteins of incubation mixtures (peaks 1, 2 and 3 in Figure 1), were pooled separately and dialyzed against 2 liters of 50 mM KH_2PO_4, containing 20% glycerol, pH 7.7 (Buffer D) overnight. After dialysis, pooled protein fractions were concentrated by ultrafiltration using PM 10 membrane (Millipore) and stored at -80°C. To characterize the proteins of these concentrated fractions, SDS-PAGE (Laemmli, 1970) and mass spectrometry were employed.

Proteolytic digestion of proteins and preparation of samples for mass spectrometry. Excised Coomassie Brilliant Blue R-250 stained protein bands from the gel of SDS-PAGE, were chopped into small cubes (approx. 1 μL) and washed several times with 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). After complete decolouration in sonication bath, the gel was washed with distilled water, shrunk by dehydration with MeCN and re-swollen again in distilled water. Thereafter, the gel was dried using a Speed-Vac evaporator and reconstituted with cleavage buffer containing 0.05 M 4-ethylmorpholine acetate, 10% MeCN and sequencing grade trypsin (Promega, 50 ng/μL) or chymotrypsin (Promega, 250 ng/μL). Digestion was carried out overnight at 37°C. The resulting peptides were extracted with 40% MeCN/0.5% acetic acid (v/v). After extraction, the peptides were directly subjected to mass spectrometry.

Mass spectrometry. Mass spectra were measured on a matrix-assisted laser desorption/ionization reflectron time-of-flight MALDI-TOF mass spectrometer UltraFLEX III (Bruker-Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm) Spectra were

calibrated externally using the monoisotopic $[M+H]^+$ ion of peptide standards PepMix I (Bruker-Daltonics). A 10 mg/mL solution of α-cyano-4-hydroxy-cinnamic acid in 50% MeCN/0.3% acetic acid was used as a MALDI matrix. A 1 μL of the sample was loaded on the target and the droplet was allowed to dry at ambient temperature and over-laid with a 0.5 μL of matrix solution. The positive MALDI-TOF spectra and LIFT MS/MS of selected ion precursors were collected in reflectron mode. MALDI-MS and LIFT MS/MS spectra were interpreted with the MASCOT program (http://www.matrixscience.com) with paying attention to preparation of electrophoresis' gel.

Incorporation of heme into apo-cytochrome b_5. The preparation of hemin chloride solution and its incorporation into apo-cyt b_5 were performed by the procedure described elsewhere (Mulrooney and Waskell, 2000). Briefly, solutions of heme were prepared by adding 3.25 mg of hemin chloride to a solution of 50% ethanol in water (5 ml) to give a final concentration of 1 mM. A small increment (10 μL) of 1 M NaOH was added and mixed to dissolve the hemin. The solution was then allowed to stand for several minutes so particulates could settle. A 10-μL aliquot was removed and diluted into 990 μL of 20 mM Tris, 1 mM EDTA, pH 8.0, at 25°C and the absorbance of the Tris-liganded heme was measured at 385 nm. The process was repeated five-times, always by adding 10 μL of 1 M NaOH, until NaOH addition caused no further increase in absorbance at 385 nm. The hemin solution was further filtered through 0.2 μm filter. Purified apo-cyt b_5 was diluted with 20 mM Tris, pH 8.0, containing 1 mM EDTA and 0.4% sodium cholate, to yield the protein concentration of 0.25 mg/ml. Aliquots of hemin chloride were added into the apo-cyt b_5 sample (1 mL) and the reconstitution of apo-cyt b_5 with heme was monitored by absorbance spectroscopy. Absorbance spectra (from 350 to 500 nm) were recorded on a Hewlett Packard 8453 UV spectrophotometer. The reconstitution was considered to be complete when the Soret peak of cyt b_5 shifted from 413 to 409 nm and the increase in absorbance at 385, caused by excess of free Tris-liganded hemin, was observed in the spectrum.

Determination of reconstituted cytochrome b_5 content. The concentration of apo-cyt b_5 reconstituted with heme was determined spectrophotometrically (the absolute absorbance spectrum) using molar extinction coefficient $\varepsilon_{413} = 117\ mM^{-1}.cm^{-1}$ (Strittmatter & Velick, 1956; Estabrook & Werringlober 1978) or from the difference spectrum of reduced minus oxidized form, using molar extinction coefficient $\varepsilon_{424\text{-}409} = 185\ mM^{-1}.cm^{-1}$, respectively (Estabrook & Werringlober 1978). The reconstituted cyt b_5 was reduced by the addition of approximately 0.2 mg of solid sodium dithionite.

Reduction of reconstituted cytochrome b_5 with NADPH:CYP reductase. The reduction of apo-cyt b_5 reconstituted with heme using NADPH:CYP reductase was carried out in liposomes as described by Enoch & Werringlober Strittmatter (1979). NADPH:CYP reductase and cyt b_5 were incorporated into liposomes prepared from DLPC as described previously (Stiborova et al. 2002, 2006). Briefly, 20 μL of a solution of DLPC in chloroform (5 mg/mL) was evaporated to dryness under nitrogen, 50 μL of 50 mM potassium phosphate buffer (pH 7.7) was added and the mixture sonicated twice for 3 minutes. The mixture was diluted with 495 μL of the same buffer, and incubated after adding 3 μM apo-cyt b_5 and/or 3 μM heme and 0.1 μM NADPH:CYP reductase for 10 minutes at room temperature and shaking at 350 RPM. The reduction reaction was initiated by adding 5 μL of 10 mM NADPH and the difference spectrum of reduced minus oxidized forms of protein was monitored on a Hewlett Packard 8453 UV spectrophotomer (from 400 to 500 nm).

RESULTS

Chromatography of proteins of the reaction mixture used for preparation of apo-cyt b_5 and their characterization with SDS-PAGE and mass spectrometry. Recently, we have found the optimal pH 4.2 for spontaneous heme transfer from cyt b_5 into apo-myoglobin, which took only 10 minutes of incubations of both proteins (Mrazova et al. 2008). In the present study, we extended our previous findings by purifying and characterizing the apo-cyt b_5 prepared by this procedure. Using chromatography on a column of DEAE-Sepharose (***Figure 1***), apo-cyt b_5 protein was purified to homogeneity (see ***Figure 2***). Apo-cyt b_5 was separated from myoglobin using chromatography on a column of DEAE-Sepharose by a step elution with 200 mM KCl in 200 mM KH_2PO_4 buffer. Using this chromatography, three major protein peaks were eluted from a DEAE-Sepharose column (Fig. 1).

Utilizing SDS-PAGE (***Figures 2 and 3***), these protein fractions were partially characterized. The results of SDS-PAGE indicate that the first protein peak corresponds to protein having molecular mass identical with myoglobin (Figure 3). The second protein peak contained protein with a relative mobility corresponding to cyt b_5, but a trace of protein with molecular mass corresponding to myoglobin seems to also present in this protein fraction (Figure 2). The protein eluted as peak 3 contained the protein with molecular mass corresponding to apo-cyt b_5 (Figure 2). These results suggest that peaks 1 and 3 correspond to myoglobin and apo-cyt b_5, respectively, whereas the protein peak 2 might correspond to a complex of both these proteins. To characterize the protein fractions further, mass spectrometry was employed in additional experiments.

The positive ion mass spectra of protein fractions were measured in the reflection mode. The values of *m/z* were manually compared after removing of matrix peaks, human keratin contamination and trypsin autoproteolytic peptides. As shown in ***Table 1***, protein fractions eluted from DEAE-Sepharose differ in several signals. The LIFT MALDI-TOF MS/MS of the

differentiated signals of peaks 1 and 2 were performed using MALDI-TOF/TOF mass spectrometer UltraFLEX (see Material and methods) with following MS/MS MASCOT program search results shown in ***Table 2*** and ***Figure 4***. These MS/MS results of peaks 1 and 2 revealed significant identification of horse heart myoglobin presence in these fractions. Moreover, the MS/MS spectrum (m/z 2279.20) of the protein peak 2 revealed the sequence that is identical with that of rabbit cyt b_5 peptide with semi-chymotrypsin cleavage or alternative C-terminal (sequence gi/159162212). Therefore, the protein peak 1 should contain myoglobin, while the protein peak 2 consists of both (apo)-myoglobin and (apo)-cyt b_5 proteins. The MS/MS spectrum of protein peak 3 (m/z 2279.20) indicates the presence of (apo)-cyt b_5 protein in this peak. These results demonstrate that chromatography on DEAE-Sepharose is a suitable procedure to separate myoglobin from apo-cyt b_5. Using such a procedure, 0.2 mg of apo-cyt b_5 from original 1.3 mg of the native rabbit cyt b_5 (16% protein yield) was obtained. Moreover, all these results indicate that a part of both proteins used for preparation of apo-cyt b_5 (apo-myoglobin and cyt b_5) remains bound in a complex (a protein peak 2 in Figure 1), which is not dissociated under the conditions used in the experiments.

Characterization of purified apo-cyt b_5 with UV/Vis spectrometry. Absorbance spectra shown in ***Figure 5*** confirmed that cyt b_5 prepared and isolated by the used procedure is in its apo-form (without heme). No heme cyt b_5 protein was present in this apo-cyt b_5 preparation (Figure 5). During the titration of the prepared apo-cyt b_5 with heme, this cofactor was readily incorporated into the apo-form of the protein to produce a holoprotein (reconstituted recombinant cyt b_5). The reconstitution of apo-cyt b_5 with heme was accompanied by color change from brown color of hemin to red bright color of the holoprotein. During formation of cyt b_5 by this procedure, an increase in absorbance at 413 nm originating from a typical Soret peak was found, which indicates that apo-cyt b_5 reconstituted with heme. The heme-reconstituted cyt b_5 protein exhibited similar physico-chemical properties such as the electrophoretic mobility on SDS-PAGE (Figure 2) and absorbance spectra of its oxidized and reduced forms as native cyt b_5 purified from rabbit livers. The Soret band at 413 nm of the oxidized form and maxima at 424, 526 and 556 nm of the dithionite-reduced form were found (***Figure 6***). In liposome vesicles, which simulate the environment of an endoplasmic reticulum membrane, purified apo-cyt b_5 reconstituted with heme can be reduced with NADPH:CYP reductase (see an increase in absorbance at 427 nm and a decrease in that at 414 nm in the spectrum shown in ***Figure 7***). The similar spectrum was found previously for native cyt b_5 purified from rabbit livers reduced with NADPH:CYP reductase (Kotrbova et al. 2009). All these findings suggest that purified apo-cyt b_5 was prepared in its native structure, without any damages in its protein function.

DISCUSSION

In this study we utilized the principle of the assay for hemin dissociation rate constant (Hargrove et al. 1994, 1996) for the preparation of the apo-hemoprotein, namely apo-cyt b_5. The method employs the fact that apo-myoglobin has a very high affinity for heme compared to other hemoproteins (Hargrove et al. 1996, Miksanova et al. 2006). For example, heme dissociation rate constant for myoglobin is 8.4×10^{-7} s^{-1} (Hargrove et al. 1996), however for the bovine cyt b_5, which is highly homologous (83.5% identity) to rabbit cyt b_5 used in this study, is $7{,}7 \times 10^{-5}$ s^{-1} (Altuve et al. 2004). Considering the fact that association of heme with apo-globin is little affected by globin structure (Hargrove et al. 1996), the heme equilibrium dissociation constant for myoglobin is at least two orders of magnitude smaller when compared to cyt b_5, showing much higher affinity to heme.

In order to prepare the apo-cyt b_5, we utilized the apo-myoglobin prepared from the horse heart myoglobin that is known to be the protein with high affinity toward the heme. The apo-cyt b_5 protein prepared by heme transfer to apo-myoglobin, was thereafter purified to homogeneity. Using the procedure described in this paper, 16% yield of apo-cyt b_5 that did not contain any heme in its molecule was obtained from the native rabbit cyt b_5. Oxidized and reduced forms of the apo-b_5 reconstituted with heme exhibit the same absorbance spectra as native cyt b_5. Moreover, the prepared apo-cyt b_5 reconstituted with heme can receive electrons from NADPH:CYP reductase. These findings indicate that a biologically active apo-cyt b_5 was prepared using transfer of heme from cyt b_5 to horse heart apo-myoglobin and its further purification. Therefore, these results suggest that the method developed in this study is suitable for preparation of apo-cyt b_5 utilized for investigation of mechanism of cyt b_5 effects on the CYP-mediated reactions. Moreover, the results found in this paper bring an interesting finding of a strong interaction of myoglobin and cyt b_5 forming a stable complex that is hard to be dissociated under the conditions used in the experiments. Now we can only speculate on a type of interactions participating in this process. Recent studies of Liang et al. (2004) investigating of electron transfer between myoglobin and cyt b_5 utilizing dynamic docking between both proteins have indicated that both proteins bind to each other by weak electrostatic interactions. Nevertheless, the results present in this study suggest that when both heme proteins compete for binding of a heme molecule, these two proteins are more tightly bound to each other. Therefore, studies explaining the mechanism of heme effects on an increase in formation of a complex of both heme proteins strongly bound to each other await further investigations.

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