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Expression and purification of the recombinant mouse tumor supressor cytochrome *b*561 protein

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ABSTRACT   It has recently been recognized that ascorbate-reducible cytochrome *b*561 (Cyt-b561) proteins constitute a well-distinguished protein family amongst the two-heme containing *b*-type cytochromes, ubiquitously present in animals and plants. Of the six isoforms that have been identified in mammals, three isoformes (called CGCytb, DCytb, and LCytb) have been cloned and expressed in yeast and/or bacterial cells. The recombinant proteins have been characterized in some detail. A particular gene product of the 3p21.3 (human) and 9F1 (mouse) chromosomal region, a so-called tumor supressor protein (101F6, TSP10), was identified as a Cyt-b561 protein by sequence homology. We have cloned and expressed the mouse tumor supressor Cyt-b561 protein (TSCytb) in yeast (*Saccharomyces serevisiae*), without and with a His$_6$-tag on either the N- or the C-terminus. The C-terminal His$_6$-tagged recombinant protein was purified on Ni-NTA His•Bind resin to almost homogeneity. Using optical spectroscopy we show that TSCytb is indeed an ASC-reducible cytochrome *b*561 protein and that ASC-reducibility is not affected by the presence of a His$_6$-tag on the C-terminus. Minor differences in the properties of TSCytb and the other three mammalian Cyts-b561 are discussed.   **Acta Biol Szeged 52(2):257-265 (2008)**

b-Type cytochromes are metallo proteins containing one or more iron:protoporphyrine-IX complexes as a chromophore. A recently discovered group of *b*-type cytochromes, called cytochromes *b*561 (Cyts-b561), consists of (i) ascorbate(ASC)-reducible, (ii) *trans*-membrane proteins with six trans-membrane helices, which (iii) have two heme *b* centers (iv) coordinated in a particular way by four well-conserved histidine residues to four consecutive *trans*-membrane α-helices (Bashtovyy et al. 2003; Tsubaki et al. 2005). The two heme *b* prosthetic groups, with different redox potential values, reside on opposite sides of the *trans*-membrane proteins making them ideal for serving as *trans*-membrane electron transporters. The Cytb-561 that was discovered first is the chromaffin granule Cyt-b561 that had already been known as chromomembrin B (Flatmark and Terland 1971; Silsand and Flatmark 1974; Apps et al. 1980). This protein functions as an electron transporter providing electrons from cytosolic ASC through the chromaffin granule membrane to intravesicular dopamine β-hydroxylase (Njus and Kelley 1993; Njus et al. 2001). After resolving the full genomic sequence of a large variety of organisms, and improving *in silico* techniques for comparing gene and protein sequences, numerous proteins were identified similar to CGCytb in various organisms, including invertebrates, vertebrates, and plants (Verelst and Asard 2003; Tsubaki et al. 2005). Three mammalian and one

plant Cyts-b561, namely the chromaffin granule Cyt-b561 (CGCytb), the duodenal Cyt-b561 (DCytb; [McKie et al. 2001]), the lysosomal Cyt-b561 (LCytb; [Zhang et al. 2006]), and the tonoplast Cyt-b561 (TCytb; [Griesen et al. 2004]), showed ASC-dependent *trans*-membrane ferrireductase activity when expressed in yeast cells (Su and Asard 2006; Bérczi et al. 2007). However, CGCytb is participating in ASC regeneration in chromaffin granules (Kent and Fleming 1987; Njus and Kelley 1993) while another isoform, DCytb, appears to be involved in extracellular recycling of ASC of human erythrocytes (Su et al. 2006). Only DCytb has already been shown to be involved in iron metabolism (McKie et al. 2001; Vargas et al. 2003). The biological function of LCytb and TCytb has not yet been elucidated.

At the level of genomic and predicted protein sequences, a human tumor supressor protein (the 101F6 gene product) has been identified as putative member of the Cyts-b561 (Lerman and Minna 2000; Ponting 2001). The mouse homologue was also discovered, sequenced, and shown to be 85% and 95% identical with the human sequences on the cDNA and protein sequence level, respectively (Lerman and Minna 2000). Both proteins have (i) 6 *trans*-membrane α-helices with (ii) 222 residues, (iii) the N- and C-termini in the cytoplasm, and (iv) 4 well-conserved histidine residues for binding two heme b prosthetic groups. 101F6 mRNA is widely expressed in animal tissues, and the mouse mRNA is especially abundant in liver, kidney, and lung (Mizutani et al. 2007), while the human protein is most abundant in liver, placenta, and lung

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Bt-CGCytb   MEGPASPARAPGALP YYVAFSQLLGLIVVAMTGAWLGMYRG GIAWESALQFNV H PLCMII  60
Mm-TSCytb   -----MALSVETESHIYRALRT ASG-AAAHLVALGFTIFVAVL ARPGSSLFSW H PVLMSL  54
Hs-TSCytb   -----MALSAETESHIYRALRT ASG-AAAHLVALGFTIFVAVL ARPGSSLFSW H PVLMSL  54

Bt-CGCytb   GLVFLQGDALLVY R----VFRNEAKRTTK VL H GLLHVFAFVIALVGLVAVF EHHRKKGYA  116
Mm-TSCytb   AFSFLMTEALLMFS PESSLLRSLSRKVRARC H WVLQLLALLCALLGLGLVIL HKEQLGKA  114
Hs-TSCytb   AFSFLMTEALLVFS PESSLLHSLSRKGRARC H WVLQLLALLCALLGLGLVIL HKEQLGKA  114

Bt-CGCytb   DLYSL H SWCGI LVFALFFAQWLVGFSFFLFPG --A SFSLRSRYRPQ H VFFGAAIFLLSVA  174
Mm-TSCytb   HLTTR H GQ AGLLAVLWAGLQCSGGMG -LL YPKLLPRWPL-AKLKLY H ATS GLVGYLLGSA  172
Hs-TSCytb   HLVTR H GQ AGLLAVLWAGLQCSGGVG -LL YPKLLPRWPL-AKLKLY H ATS GLVGYLLGSA  172

Bt-CGCytb   TALLGL KEALLFELGTKYSMFEPE GVLANVLGLLLATFATVILYIL TRADWKRPLQAEEQ  234
Mm-TSCytb   SLLLGMFS -LW FTAT VTGGAWY -LAVLCPILTSLVI MNQVSNAYL-----YRKRIQP---  222
Hs-TSCytb   SLLLGMCS -LW FTAS VTGAAWY -LAVLCPVLTSLVI MNQVSNAYL-----YRKRIQP---  222

Bt-CGCytb   ALSMDFKTLTEGDSPSSQ 252
Mm-TSCytb   ------------------
Hs-TSCytb   ------------------
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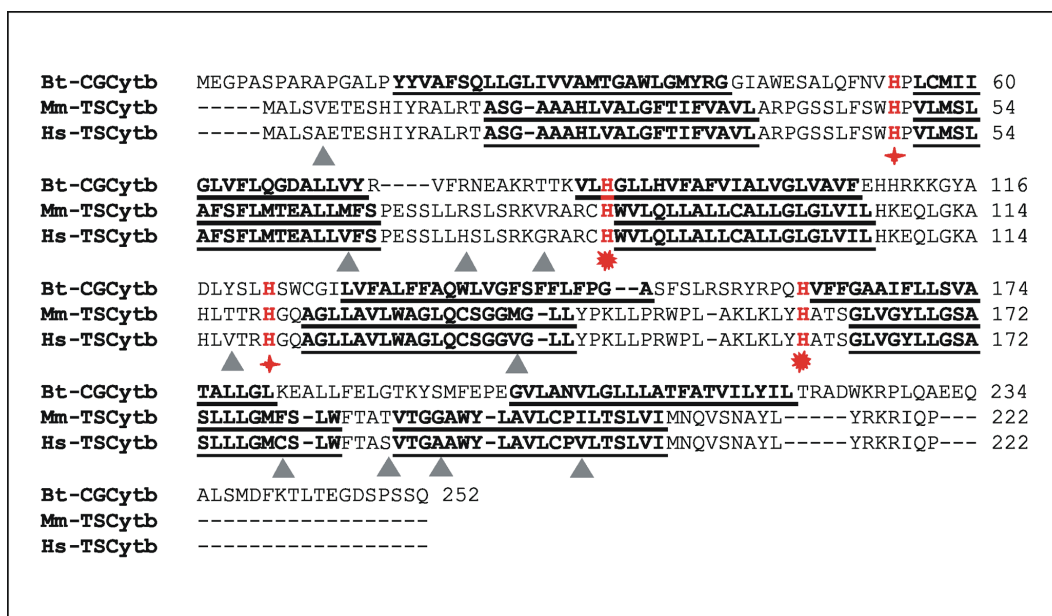
Figure 1. Multiple alignment of bovine (*Bos taurus*, Bt) CGCytb, mouse (*Mus musculus*, Mm) TSCytb, and human (*Homo sapiens*, Hs) TSCytb using Clustal W (v. 2.0.1) software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Predicted trans-membrane segments are bold faced, underlined, and obtained by using HMMTOP (http://www.enzim.hu/hmmtop/). The highly conserved His residues binding the two hemes are labelled in pairs with stars (* and +). Gray triangles (▲) show the 10 amino acid residues where the mouse and human TSCytb are different.

(Lerman and Minna 2000). Expression of wild-type101F6 in tumor cells significantly inhibited cell growth and intra-tumoral injection of recombinant adenovirus-101F6 gene vectors, as well as systemic administration of protamine-complexed adenovirus-101F6 gene vectors, significantly suppressed tumor xenografts growth (Ji et al. 2002). Ohtani et al. (2007) have recently found that nanoparticle-mediated 101F6 gene transfer and a subpharmacologic concentration of ASC synergistically and selectively inhibited tumor cell growth by caspase-independent apoptosis and autophagy *in vitro* and *in vivo*. C terminal *myc*-tagged mouse 101F6 protein has been expressed in Chinese hamster ovary (CHO) cells and immunofluorescence microscopy was used to lo-calize the recombinant proteins; they were found in small vesicles, including endosomes and endoplasmic reticulum of the perinuclear region (Mizutani et al. 2007). It was also shown that CHO cells expressing the C terminal *myc*-tagged mouse 101F6 protein showed higher ferric ion and azo-dye reduction level than the control CHO cells. Mizutani et al. (2007) concluded that mouse 101F6 proteins played roles in the ferrireduction via a yet unresolved mechanism.

In this paper we report on the cloning and expression of the mouse 101F6 gene in a yeast expression system, both with and without a His_6-tag on either the C-terminus or the N-terminus. It is shown that the recombinant mouse tumor suppressor protein is indeed an ASC-reducible *b*-type cyto-chrome and the His_6-tag on the C-terminus does not affect ASC-reducibility.

Materials and Methods

Chemicals

The protease inhibitor cocktail tablet „cOmplete", NBT (Nitro blue tetrazolium chloride), BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) in 67% DMSO (v/v), were from Roche (Mannheim, Germany); Sucrose monolaurate (SML) was from Dojindo (Tokyo, Japan); Ni-NTA His•Bind Resin was from Novagen (Darmstadt, Germany); Difco™ yeast nitrogen base w/o amino acids was from BD (Sparks, MD, USA); other chemicals were analytical grade and from Acros (Geel, Belgium) or Fluka (Buchs, Switzerland) or Serva (Heidelberg, Germany).

Molecular biology works

For expression of untagged proteins, the cDNA of mouse TSCytb (the sequence corresponds to GenBank protein entry, NP_062694; see Fig. 1) was amplified from mouse whole brain RNA and was cloned into a pESC-His expres-sion vector (Stratagene, La Jolla. CA), downstream of the GAL10 galactose-inducible promoter, using EcoRI and SpeI restriction sites. Yeast cells (*Saccharomyces cerevisiae*, strain YPH499: ura3-52 lys2-801[amber]ade2-101[ochre] trp1-Δ63 his3-Δ200 leu2-Δ1) were transformed and grown according to the manufacturer's instructions (Stratagene).

For expression of His_6-tagged proteins, a standard PCR method was used to amplify the gene for TSCytb from mouse

whole brain RNA. Primers were designed to generate EcoRI and SpeI sites for cloning into the pESC-His expression vector (Stratagene, La Jolla, CA), downstream of the GAL10 galactose-inducible promoter, and with a His$_6$ tag either on the C- or the N-terminus. Four oligonucleotide primers were synthesized and used for the amplification:

C-terminus, sense: 5'-GGGGGAATTCGCCACCATG-GCCCTTTCTGTGGAGACG-3'

C-terminus, antisense:
5'-CCCCACTAGTCTAGTGATGGTGATGATGGTGT-GGCTGGATCCTCTTGCGGT-3'

N-terminus, sense:
5'-GGGGGAATTCACCATGCACCATCATCACCAT-CACGCCCTTTCTGTGGAGACGGAG-3'

N-terminus, antisense: 5'-CCCCACTAGTTCATGGCT-GGATCCTCTTGCGGT-3'

First, the PCR-amplified products were inserted into pGEM-T Easy vectors (Promega Corp., Madison, WI, USA), and the resulting vectors were transformed into *E. coli* DH5α cells using calcium chloride competent cells and the Nucleo-Spin Plasmid QuickPure kit (Macherey-Nagel GmbH & Co., Düren, Germany). The pGEM-T Easy vectors with the inserts were isolated with the NucleoSpin Plasmid QuickPure kit and inserted into empty pESC-His vectors by using the restriction enzymes EcoRI and SpeI. The integrity of the His$_6$-tagged TSCytb/pESC-His vectors was confirmed by restriction digestion and DNA sequencing. Finally, pESC-His verctors were transformed into competent yeast cells (*Saccharomyces cerevisiae*, strain YPH499, ura3-52 lys2-801[amber]ade2-101[ochre] trp1-Δ63 his3-Δ200 leu2-Δ1) and the transformed cells were tested on SD-dropout medium (http://www.stratagene.com/manuals/217451.pdf) where the lack of histidine was the selective factor. For induction of protein expression and production of the recombinant proteins, overnight cultures were grown in SD-dropout medium, transferred to SG-dropout medium (http://www.stratagene.com/manuals/217451.pdf) and grown for 24-28 hours.

Yeast membrane preparation

Cells grown as 2 x 350 ml cultures in SG-His medium were collected by low-speed centrifugation (at 4,000 g_{max} and 10°C for 10 min) when the OD_{600}(d=1cm) reached a value of 0.8 ± 0.05. Collected cells were washed once with ice cold phosphate buffer (20 mM KH_2PO_4, 100 mM NaCl, pH 7) and pelleted as above. Washed cells were resuspended in 15 ml of ice cold homogenization buffer (50 mM MES-KOH, pH 7.0, 5 mM EDTA, 150 mM KCl, 150 mM sucrose) supplemented with freshly added 0.1% (w/v) Na-ASC and protease inhibitors (1 mM PMSF and aprotinin, pepstatin, leupeptin, antipain, chymostatin in the form of 1 dissolved cOmplete tablet). Cells were broken in a Bead Beater (Biospec Products, Bartlesville, OK, USA) with four 30 s cycles, with 2 min cooling intervals, using 0.5 mm glass beads. Beads were

rinsed with 35 ml of ice cold homogenization buffer. The homogenate (50 ml) was centrifuged at 4,000 g_{max} and 5°C for 10 min to remove unlysed cells and cell debris. A yeast microsomal membrane (YM) fraction was obtained after centrifugation of the 4,000 g_{max} supernatant at 75,000 g_{max} and 4°C for 60 min (Sorvall Discovery 90 ultracentrifuge and T-647.5 rotor; Sorvall Products, L.P., Newtown, CT, USA). After centrifugation, the pellet was resuspended in 20 mM phosphate buffer, pH 7, and the membrane vesicles were stripped as before (Bérczi et al. 2005). Stripped microsomal membrane (SYM) vesicles were resuspended in phosphate buffer (50 mM NaH_2PO_4, 10% (w/v) glycerol, pH 7), and stored at −80°C until use.

Sucrose density gradient fractionation

For membrane fractionation, after stripping and final centrifugation, SYM vesicles were resuspended in 4 ml of MES-KOH buffer (50 mM MES, 10% (w/v) sucrose, pH 7) and layered on the top of a discontinuous sucrose density gradient made of 2 ml of 70% (w/v), 10 ml of 45% (w/v), 9 ml of 32% (w/v), and 8 ml of 20% (w/v) sucrose solutions in 50 mM MES-KOH buffer, pH 7. Fractionation was by centrifugation in an AH-629 swing-out rotor (Sorvall Products, L.P., Newtown, CT, USA) in a Discovery 90 ultracentrifuge at 28,900 rpm (g_{max}=150,000g), at 5°C for 3 hours. The cloudy bands at the interfaces were collected with a Pasteur pipette, diluted ten-fold by 20 mM MES-KOH, pH 7, and pelleted by centrifugation (Sorvall T-647.5 rotor and Discovery 90 ultracentrifuge) at 75,000 g_{max} and 4°C for 90 min. After centrifugation, the pellet was resuspended in phosphate buffer (50 mM NaH_2PO_4, pH 7, containing 10% (w/v) glycerol), and stored at −80°C until use.

Protein solubilization and purification by His$_6$-tag affinity chromatography

Frozen membrane vesicles (about 60 mg protein) in 50 mM NaH_2PO_4, 10% (w/v) glycerol, pH 7, were thawn and proteins were solubilized by sucrose monolaurate (SML) at 1 mg ml^{-1} protein concentration and 3:1 (w/w) detergent:protein ratio. The mixture was incubated on a rocker at 5°C for 90 min. Insoluble material was pelleted by high-speed centrifugation (T-647.5 rotor and Sorvall Discovery 90 ultracentrifuge) at 75,000 g_{max} and 5°C for 60 min. The supernatant containing the detergent-solubilized proteins was supplemented with 500 mM NaCl and 10 mM imidazole, the pH was adjusted to 7.8 and then mixed with 1 ml (bed volume) of Ni-NTA His•Bind resin (Novagen, Madison, WI, USA) that had been pre-equilibrated with 50 mM NaH_2PO_4, pH 7.8, containing 10% (w/v) glycerol, 1%(w/v) SML, 500 mM NaCl and 10 mM imidazole. The affinity resin was incubated with the solubilized proteins at room temperature for 30 min. After incubation, the Ni-NTA His•Bind resin with bound proteins was collected in a 10-ml disposable column and washed 3
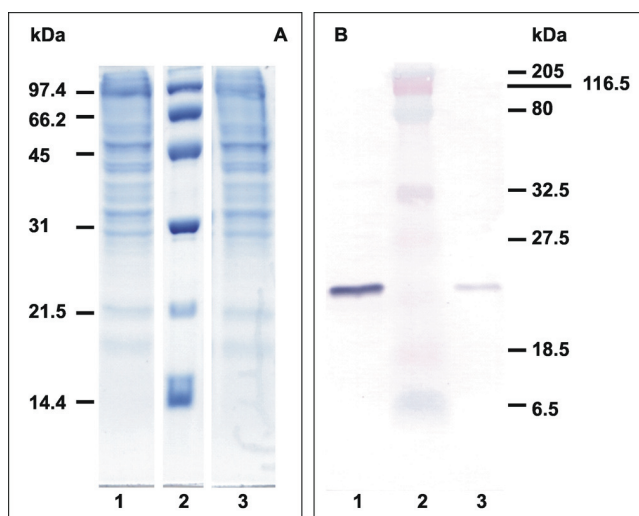
Figure 2. Coomassie Brillant Blue R-250 stained (A) and Western blotted (B) SDS-PAGE picture of YM fractions containing C-terminal (lane 1) or N-terminal (lane 3) His$_6$-tagged TSCytb. Molecular mass standards are on lanes 2. 25 µg (A) and 10 µg (B) of proteins were loaded.

spectrum was obtained at room temperature. When improvement of the signal to noise ratio was needed, multiple scans were averaged. Amount of cytochromes was calculated from the reduced-minus-oxidized difference spectra by using a millimolar extinction coefficient of $\varepsilon_{561nm}=30$ mM$^{-1}\cdot$cm^{-1} (Tsubaki et al. 1997; Liu et al. 2005).

Gel electrophoresis and Western blotting

Protein content of samples (both of membranes and of detergent micelles) was estimated by the modified Lowry method (Markwell et al. 1978) with deoxycholate as detergent and BSA as standard.

Proteins were resolved by SDS-PAGE (Laemmli 1970) by using 4.5% (stacking) and 12% (running) polyacrylamide gels. Samples were not heated or boiled prior to loading on gels because harsh temperature treatment is known to cause the highly hydrophobic integral membrane proteins to aggregate, preventing them from penetrating into the 12% running gel (Duong and Fleming 1982; Thomas and McNamee 1990). Low Range and Kaleidoscope Prestained molecular weight standards (BioRad, Hercules, CA, USA) were used for the Coomassie staining and for the Western blotting, respectively.

For protein visualization, gels were stained with 0.2% (w/v) Coomassie Brillant Blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic acid for 2 hours and destained in 40% (v/v) methanol, 10% (v/v) acetic acid in three 30 min steps. Finally the destained gels were rinsed in 40% (v/v) methanol, 10% (v/v) acetic acid, 5% (v/v) glycerol and dried between two layers of wet cellophane sheets at room temperature.

For visualization and identification of His$_6$-tagged TSCytb, gels were transferred onto polyvinylidene difluoride membranes (PVDF) with a Mini Trans-Blot Electrophoretic Transfer Cell (both from BioRad, Hercules, CA, USA) in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol. A mouse monoclonal antibody to the 6X-His tag (Abcam, Cambridge, UK) was used to detect the recombinant proteins. Protein-antibody complexes were visualized by alkaline phosphatase-coupled rabbit-anti-mouse secondary antibodies (Pierce, Product #: 31332, Rockford, IL, USA) after reacting with NBT/BCIP (http://www.cshprotocols.org/cgi/content/full/2007/15/pdb.rec11089).

All results shown are representative from 2 to 4 independent repetitions.

Results and Discussion

Expression and purification

The YPH499 yeast cell line and pESC-His vector has already been successfully used for cloning His$_6$-tagged Cytbs-561 (Griesen et al. 2004; Bérczi et al. 2005; Zhang et al. 2006) and the expression system provided recombinant CGCytb with physico-chemical properties similar to the native pro-
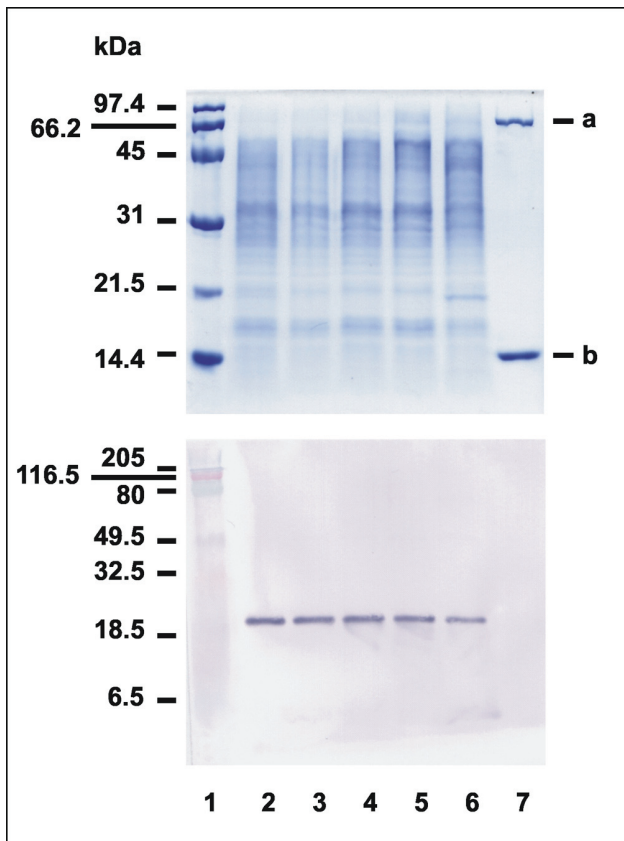
times with 5 bed volumes of 50 mM NaH$_2$PO$_4$, pH 7.8, containing 10% (w/v) glycerol, 0.3% (w/v) SML, 500 mM NaCl and 10 mM imidazole. His$_6$-tagged proteins were eluted with 4 bed volumes of elution buffer (50 mM NaH$_2$PO$_4$, pH 7.0, containing 10% (w/v) glycerol, 0.3 %(w/v) SML, 150 mM NaCl and 250 mM imidazole). The affinity resin was further washed with 10 bed volumes of elution buffer while the eluate was concentrated by centrifugation using Centricon-YM100 centrifugal filter unit (Millipore, Bedford, MA, USA) and desalted by using a PD-10 desalting column (Amersham Biosciences, Uppsala, Sweden). The desalted fraction was concentrated again by centrifugation using Centricon-YM100 centrifugal filter units and the concentrated fraction was stored in 50 mM NaH$_2$PO$_4$, 10% (w/v) glycerol, 0.3 % (w/v) SML, pH 7, at −80°C until use.

Absorption spectroscopy

Absorption spectra with SYM vesicles were recorded in dual wavelength mode between 500 nm and 600 nm (with 601 nm as reference wavelength) while absorption spectra with the solubilized protein fractions were recorded in split beam mode between 350 nm and 650 nm (with buffer as reference) by using an OLIS-updated SLM-Aminco DW2000 spectrophotometer (OLIS Co., Bogart, GA, USA) with 2 nm slit-width at room temperature and under continuous stirring. First, cytochromes were oxidized by addition of ferricyanide (0.1-0.2 mM, K$_3$[Fe(CN)$_6$]) and the fully oxidized spectrum was recorded. Then ASC was added and the ASC-reduced spectrum was recorded. Finally Na-dithionite (DTH) was added (2-5 mM final concentration) and the DTH-reduced

Figure 3. Coomassie Brillant Blue R-250 stained (top) and Western blotted (bottom) SDS-PAGE picture of SYM fractions containing (H_6C)TSCytb. Lanes are: 1 for molecular mass standards, 2 for SYM, 3 for SYM at the 10%/20% sucrose step interface, 4 for SYM at the 20%/32% sucrose step interface, 5 for SYM at the 32%/45% sucrose step interface, 6 for SYM at the 45%/70% sucrose step interface, and 7 for BSA and cytochrome c. 18 µg (top) and 2 µg (bottom) of protein-were loaded in lanes 2 through 6. a - 66.2 kDa for BSA; b - 12.4 kDa for horse cytochrome c.
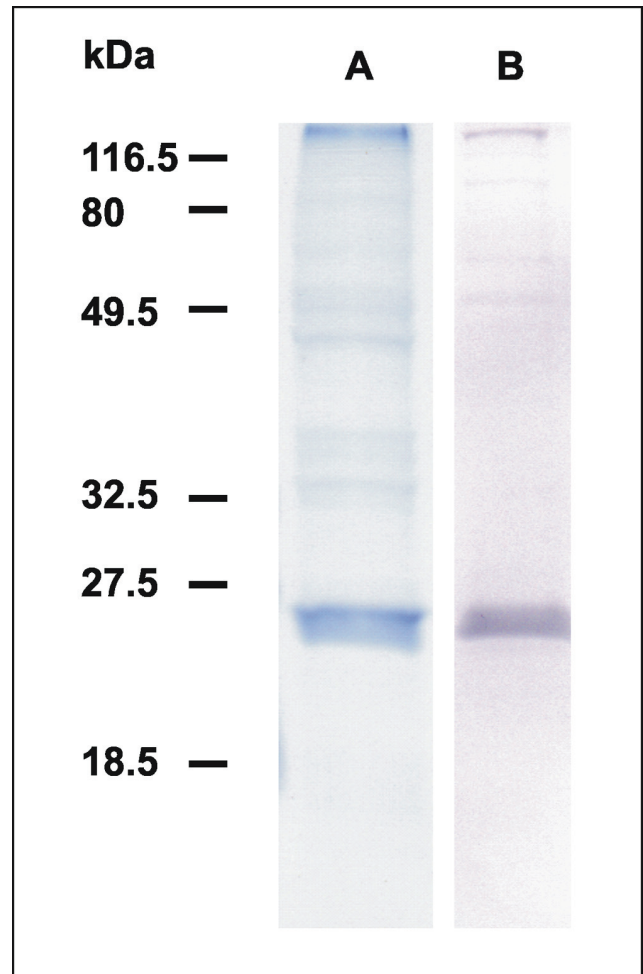

Figure 4. Coomassie Brillant Blue R-250 stained (A) and Western blotted (B) SDS-PAGE picture of highly purified (H_6C)TSCytb after PD-10 chromatography and concentration. 13 µg (A) and 3 µg (B) of proteins were loaded.

tein (Bérczi et al. 2005). Other yeast cell lines and vectors were also successfully employed in high yield expression of mammalian Cyts-b561 (Liu et al. 2005; Su and Asard 2006). It was therefore plausible to use a yeast expression system for producing appropriate amounts of a novel recombinant Cytb-561 and establishing the basic physico-chemical properties of the expressed protein. N-terminal or C-terminal His_6-tagged TSCytb was expressed and the YM fraction was used to check the expression levels. Figure 2 shows that the concentration of the N-terminal His_6-tagged TSCytb in the YM fraction was considerably lower than that of the C-terminal His_6-tagged TSCytb (hereafter (H_6C)TSCytb). This result is in agreement with former observations obtained with the His_6-tagged CGCytb and TCytb proteins (data not published), although the reason of the difference in expression levels is not yet known.

When mammalian or plant Cyts-b561 were expressed in ferrireductase-deficient yeast cells (*Δfre1Δfre2* mutant lines), the capacity of these cells to reduce extracellular ferric-compounds was restored (Su and Asard 2006; Bérczi et al. 2007). These results were indirect proof that the recombinant Cyts-b561 was at least in part, targeted to the yeast cell plasma membrane.

Continuous sucrose density gradient fractionation of YM vesicles revealed that plasma membrane vesicles assembled at position of rather high sucrose concentrations (>45% (w/v); Roberg et al. 1997). When the distribution of (H_6C)TSCytb in the SYM fraction was studied by discontinuous sucrose density gradient centrifugation and Western blotting, (H_6C)TSCytb was almost evenly distributed in membrane fractions at all sucrose density steps (Fig. 3). In fact, the lowest concentration of TSCytb appears at the highest sucrose

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Table 1. Protein balance sheet. For each His$_6$-tag affinity chromatography step, 100S fractions from two independent experiments were used; for each solubilization step, SYM fractions from two independent membrane preparations were used; and at each membrane preparation, 700 ml of yeast cell cultures with OD$_{600}$(d=1 cm) of 0.8±0.05 was used. Results are the means ± s.d. from 8 independent His$_6$-tag affinity chromatography steps except for the last value (§) which is mean ± s.d. from 4 independent purifications. YM – yeast microsomal membranes, SYM – stripped yeast microsomal membranes, 100P – unsolubilized membranes (the 100,000 g$_{max}$ pellet after the solubilization step), 100S – the concentrated protein fraction after solubilization, Filtrate – solubilized protein fraction which passed through the 100,000 kDa membrane filter, Unbound – solubilized proteins unbound to the Ni-NTA His•Bind Resin, (H$_6$C)TSCytb – concentrated His$_6$-tagged TSCytb proteins eluted from the Ni-NTA His•Bind Resin. n.m. is for "not measured".

Fraction	Total protein, mg	ASC-reduced TSCytb pmol·mg^{-1}
YM	198 ± 47	n.m.
SYM	143 ± 31	77± 18
100P	72 ± 15	n.m.
100S	60 ± 13	n.m.
Filtrate	5.7 ± 1.4v	n.m.
Unbound	46 ± 7	n.m.
(H$_6$C)TSCytb	1.2 ± 0.3	3850 ± 341 [§]

Figure 5. ASC-reduced (a) and dithionite-reduced (d) minus oxidized difference spectra of SYM fraction containing untagged (A) and His$_6$-tagged (B) TSCytb. 7.5 mg·ml^{-1} was the protein concentration in both cases.

concentration step employed, *i.e.* the location at which the yeast plasma membrane is expected. Mizutani et al. (2007) showed that myc-tagged TSCytb was localized in small vesicles, including endosomes and endoplasmic reticulum of the perinuclear region, using an immunofluorescent staining technique. Therefore, the localization of (H$_6$C)TSCytb in the lighter SYM fractions was not a surprise. However, the rather homogeneous distribution of (H$_6$C)TSCytb in different and lighter populations of yeast membrane vesicles appears to be different from the mostly yeast plasma membrane localization of the other three recombinant mouse Cyts-b561 expressed in yeast. This result pointed out that enrichment of a membrane fraction containing (H$_6$C)TSCytb by differential and/or density gradient centrifugation before membrane solubilization could not be achieved.

As the protein balance sheet shows (Table 1), stripping of the YM vesicles resulted in the loss of about 30% of the proteins. Treatment of the SYM vesicles with the nonionic detergent SML, at a detergent:protein ratio of 3:1 (w/w) and 1 mg/ml protein concentration, resulted in solubilization of about 45% of the membrane proteins. When the solubilized fraction was concentrated by using a membrane filter with 100 kDa cut-off value, >90% of solubilized proteins were retained. About 75% of the solubilized and concentrated proteins did not bind the Ni-NTA His•Bind resin (Table 1, unbound fraction), however, only about 8% of the bound proteins were strongly associated with the resin, and eluted using the elution buffer (Table 1, (H$_6$C)TSCytb fraction). In order to exchange the high salt and imidazole-containing
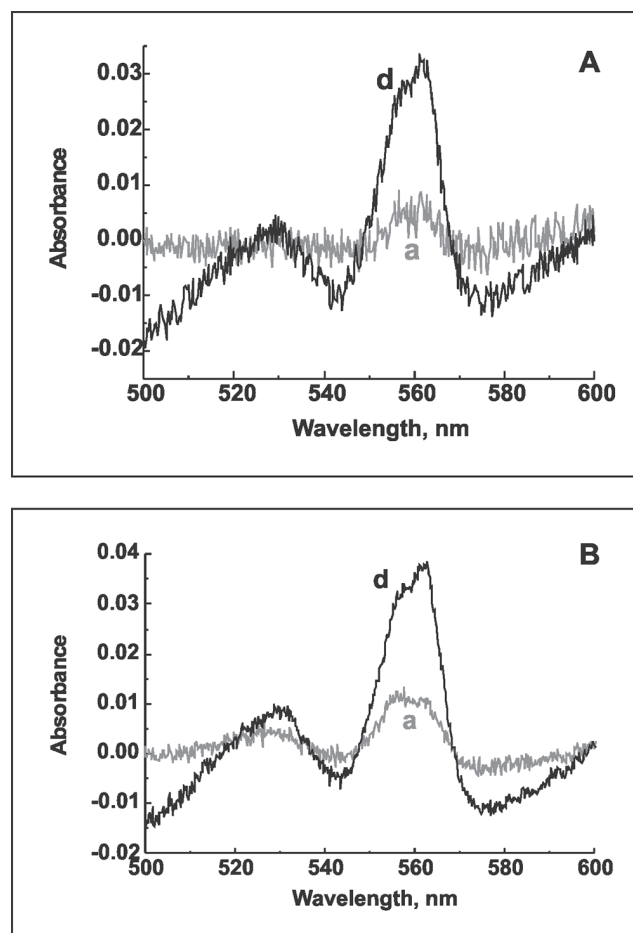
elution buffer to a widely-used phosphate buffer for storage of (H$_6$C)TSCytb, the coral red fraction was passed through a PD-10 column and then concentrated. Figure 4 shows that the final (H$_6$C)TSCytb fraction contained mostly His$_6$-tagged TSCytb, but small amounts of contaminating proteins can be seen on the gel. Even the fraction of proteins, which did not enter the separation gel, contained (H$_6$C)TSCytb, according to the Western blot staining. After solubilization, protein concentration, affinity chromatography, PD-10 chromatography, and a second protein concentration step, the ASC-reducible TSCytb recovery was between 40 and 50% which is in good agreement with former results (Bérczi et al. 2007).

Spectroscopy

TSCytb was classified as member of the Cyts-b561 on the basis of its sequence homology and predicted structural similarity to bovine CGCytb (Ponting 2001; Tsubaki et al. 2005; Su and Asard 2006). Figure 5 shows that TSCytb is
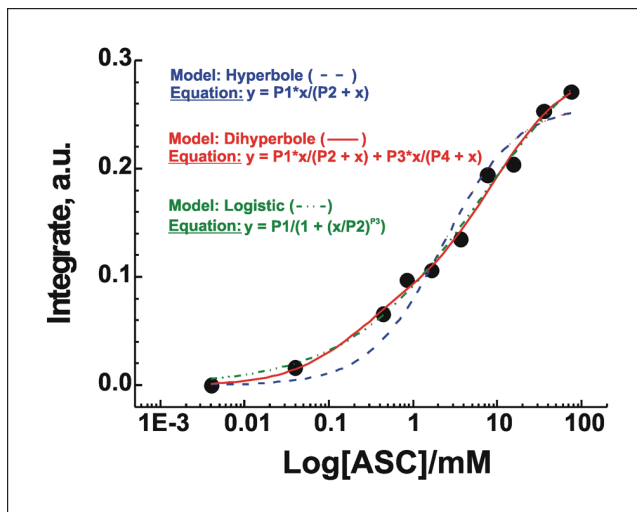
Figure 6. ASC-dependent reduction of highly purified (H$_6$C)TSCytb. Protein concentration was 0.4 mg·ml^{-1} Y axes are values obtained by integration between 542 and 569 nm of the ($\alpha+\beta$)-peak in the reduced minus oxidized difference spectrum. Experimental points cannot be fitted by a single ASC-binding or -affinity site model (Hyperbole fit, dash line, r^2<0.95). However, both a two ASC-binding or -affinity site model (Dihyperbole fit, solid line) and a „logistic-type" model (Logistic fit, dash-dot-dot line) gave appropriate fits with similar r^2 values (>0.98). Mathematical analysis was with OriginPro 7.5 Data Analysis and Graphing Software.

Figure 7. Oxidized, ASC-reduced and dithionite-reduced spectra of highly purified (H$_6$C)TSCytb. Protein concentration was 0.4 mg·ml^{-1}.

indeed a membrane-associated *b*-type cytochrome, with an α-band absorbance maximum around 560 nm. TSCytb is also ASC reducible which is the basic property of Cyts-b561. It is evident that the presence of a His$_6$-tag on the C terminus does not affect the ASC reducibility of the protein. However, the specific ASC-reducible cytochrome *b* content of SYM vesicles (between 50 and 100 pmol·mg^{-1} protein), tested with 25 mM ASC, was much lower than that obtained with other Cyts-b561 expressed in yeast (CGCytb: about 1500 pmol.mg^{-1} [Bérczi et al. 2005]; TCytb: about 400 pmol.mg^{-1} [Bérczi et al. 2007]; LCytb: about 150 pmol.mg^{-1} [Zhang et al. 2006]). It is also evident that the untagged and His$_6$-tagged protein in the SYM fraction had considerably lower ASC reducibility, as compared to dithionate reducibility, than either CGCytb or TCytb (see later).

Recombinant CGCytb and TCytb reached saturation in ASC-reducibility at 25 mM ASC (Lakshminarasimhan et al. 2006; Bérczi and Asard 2006). However, this was not the case with TSCytb. As seen in Figure 6, ASC-reduction continued to increase even at 75 mM ASC. It is evident that the ASC-dependent reduction of TSCytb cannot be described by a single binding or affinity site, as it was also evident with CGCytb and TCytb (Lakshminarasimhan et al. 2006; Bérczi and Asard 2006). However, the two binding or affinity site model, which could explain the ASC-dependent reduction of CGCytb and TCytb, cannot be unambiguously employed for TSCytb due to the uncertainty of experimental points; as seen
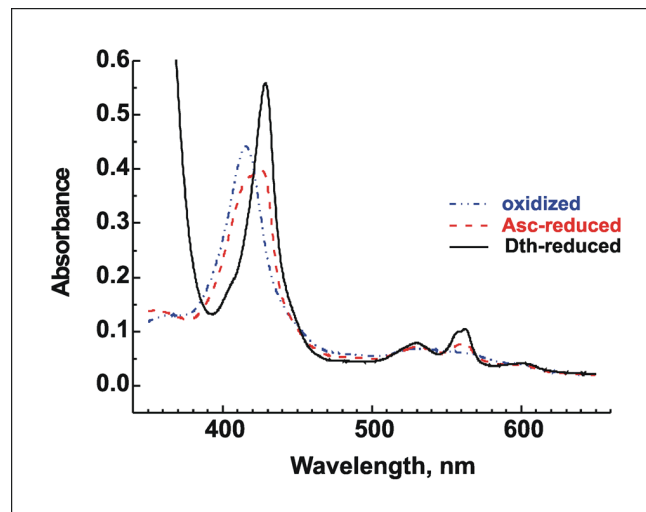
in Figure 6, both a "two binding site model" and a "logistic model" provide fitted curve with r^2>0.98.

Finally, maximal reduction of *b*-type cytochromes can be obtained in the presence of 2-5 mM dithionite. The maximal ASC-reduction of CGCytb and TCytb is about 75-80% of the dithionite-reduced maximal reduction value (Bérczi et al. 2005, 2007). As Figure 7 shows, reduction of highly purified TSCytb at 50 mM ASC concentration is only about 50% of that at 5 mM dithionite concentration. This is particularly well seen in the Soret band of spectra where the disappearance of the absorption peak at 415 nm in the fully oxidized spectrum is parallel with the appearance of the γ-peak at 428 nm in the fully reduced spectrum through an isosbestic point at 421 nm.

The specific cytochrome *b* content of the highly purified (H$_6$C)TSCytb fraction was about 4 nmol·mg^{-1} protein (Table 1). This value is again lower than (one third of) those values obtained with highly purified recombinant mouse (H$_6$C) CGCytb and (H$_6$C)TCytb (Bérczi et al. 2005, 2007) but the difference is smaller than that observed at the level of SYM vesicles (see above). One reason for the difference between the specific TSCytb content in the SYM fraction and highly purified protein fraction could lie in different expression levels of the different recombinant proteins in yeast. However, another source for the observed differences could be that in all calculations we use the molar extinction coefficient established for the bovine CGCytb at 561 nm and with dithionite as the reducing agent. It is possible, on the basis of differences in the primary structure of Cyts-b561 (see Fig. 1), that different Cyts-b561 have different molar extinction coefficients. Furthermore, detailed reduction mechanisms with ASC and/or dithionite have not yet been established. It is well known that the presence of dithionite in solutions causes

oxygen depletion. Oxygen depletion is transient, depending on the starting concentration of dithionite and contact of the solution with air (that is mostly the case in spectrophotometer cuvettes). Since the role of oxygen as an oxidant to Cyts-b561 is not yet clear, it may affect the final reduction levels of the protein when any reducing agent is added. Taken together, we conclude that a reliable quantitative comparison between the properties of different Cyts-b561 will be possible only after establishing the proper molar extinction coefficient for each protein and the role of disolved oxygen for the reduction mechanism(s) of Cyts-b561.

Comparison of TSCytb to CGCytb and TCytb

In this paper, we have reported the expression of the His_6-tagged recombinant mouse TSCytb in the very same yeast expression system previously used for mouse CGCytb, DCytb, LCytb, and TCytb from Arabidopsis thaliana. It is shown that (i) TSCytb is an ASC-reducible *b*-type cytochrome but its sensitivity to ASC is considerably lower than that observed with CGCytb and TCytb, (ii) the specific content of TSCytb is lower than those of CGCytb and TCytb in the very same SYM fractions, (iii) the ASC-reducibility of TSCytb is lower than that of CGCytb and TCytb when compared to the maximal reduction of these Cyts-b561 by dithionite. These minor differences in the basic ASC-reducible properties might be explained by detailed sequence comparison of these proteins. Indeed, it was found that CGCytb, DCytb, LCytb, and even TCytb are evolutionary much closer to each other than to TSCytb (Tsubaki et al. 2005; Su and Asard 2006), supporting that these proteins might have little but important differences in structures which is manifested in different physico-chemical properties.

Acknowledgements

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