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Substrate-dependent reduction of a recombinant chromaffin granule Cyt-*b*561 and its R72A mutant

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ABSTRACT Cytochrome *b*561 (Cyt-*b*561) proteins constitute a family of integral membrane proteins, catalyzing ASC-driven trans-membrane electron transport. Numerous isoforms of Cyt-*b*561 are present in invertebrates, vertebrates, and plants. The only protein of this family, however, which has been characterized in details at both biophysical, biochemical and physiological levels so far, is the bovine chromaffin granule Cyt-*b*561 (CGCyt-*b*561). Recently, both the bovine and the mouse CGCyt-*b*561 has been expressed in yeast cells and the recombinant proteins were shown to have biophysical properties similar to the native bovine CGCyt-*b*561. We have expressed the mouse CGCyt-*b*561 with a His_6-tag at the C terminus (CGCyt-*b*561(C6H)) in yeast (*Saccharomyces serevisiae*) cells and studied the reduction of CGCyt-*b*561(C6H) in the presence of different natural reducing agents. Besides the well-known natural reductant ascorbate (ASC) and the often-used artificial reductant dithionate, NADH, GSH, and dihydrolipoic acid (DHLA), also reduced the fully oxidized protein. Interestingly however, NADPH was not effective at all. When the same reductants were tested with the R72A mutant of CGCyt-*b*561(C6H), a mutant with impaired ASC-dependent reducibility, neither pyridine-dinucleotides could reduce the R72A mutant. DHLA-dependent and ASC-dependent reduction kinetics were very similar in case of the R72A mutant but differed in case of CGCyt-*b*561. These results raise the question of how many natural reductants the CGCyt-*b*561 may utilize *in vivo*.
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KEY WORDS

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His_6-tagged protein
cytochrome b561
reductants

Cytochromes *b*561 (Cyts-*b*561) constitute a newly described family of membrane proteins, present in both plants and animals (Asard et al. 2001; Tsubaki et al. 2005). Cyts-*b*561 contain two *b*-type heme molecules per molecule of protein (Tsubaki et al. 1997). There are 6 trans-membrane (TM) α-helices in Cyts-*b*561 and TM2 through TM5 define the so-called CB domain (Pointing 2001). One His residue on each α-helix of the CB domain is highly conserved and participates in coordination of the two hemes (Okuyama et al. 1998). Structure prediction studies indicate that, CGCyt-*b*561 follows the "Four-helix motif" (Lancaster 2002), where the extravesicular heme is coordinated by His residues on TM3 and TM5, and the intra vesicular heme is coordinated by His residues on TM2 and TM4 (Bashtovyy 20003).

Cyt-*b*561 of the chromaffin granules (CGCyt-*b*561) is the best-characterized member of the Cyt-*b*561 protein family. CGCyt-*b*561 is able to mediate trans-membrane electron transfer from ASC present on the extra-vesicular side to monodehydroascorbate (MDHA) present on the intra-vesicular side of the protein (Kent and Fleming 1987; Fleming and Kent 1991). Two regions of CGCyt-*b*561 have been identified as putative substrate binding sites, the partially conserved region ^{69}ALLVYRVFR^{74} (numbering is for the bovine CG-Cyt-*b*561) present at the end of TM2 helps in ASC binding while the well-conserved motif of ^{120}SLHSW^{125}, present on the intra-vesicular side of CGCyt-*b*561 presumably aids in binding of MDHA. Chemical modification studies on bovine CGCyt-*b*561, by treating the protein with diethyl-pyrocarbonate (DEPC) showed the importance of His residues in mediating trans-membrane electron transfer. DEPC has the ability to interact with His, Lys and Tyr residues and covalently modify them. CGCyt-*b*561, treated with DEPC, had altered properties with respect to substrate binding. Mass spectrometry revealed that H88, H159, and K85 in the bovine CGCyt-*b*561 were modified. To His residues were assigned a role in the coordination of extra-vesicular heme and K85 was thought to be involved in ASC binding (Tsubaki et al. 2000). Recent studies with recombinant mouse CGCyt-*b*561 revealed the importance of an Arg residue (R72 in the mouse CGCyt-*b*561 sequence) in ASC-dependent reduction of the protein as well as identified the essential His residues coordinating the two hemes and responsible for the optical properties of CGCyt-*b*561 (Bérczi et al. 2005).

In the near past, new members of the Cyt-*b*561 protein family were identified. One such protein is the so-called duodenal Cyt-*b*561 (DCyt-*b*561) was identified by subtractive cloning strategy (McKie et al. 2001). DCyt-b561 was shown to localize at the brush border membranes of the duodenum

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and exhibited ferric reductase activity when transiently expressed in Xenopus oocytes and in intestinal cell lines (McKie et al. 2001). Other new member of the Cyt-*b*561 protein family includes the lysosomal Cyt-*b*561 (LCyt-*b*561), named after its localization in lysosomes of macrophages and sertoli cells. LCyt-*b*561 is similar to CGCyt-*b*561 in spectral characteristics and is possibly involved in iron metabolism (Deliang Zhang and Han Asard, personal communication). A fourth isoform, tentatively named "tumor suppressor Cyt-*b*561" (gene name:101F6, herein abbreviated as TSCyt-*b*561) was identified in the 120kb tumor homozygous deletion region of chromosome 21 found in breast and lung cancers (Ji et al. 2002). Over-expression of TSCyt-*b*561 inhibited tumor cell growth by induction of apoptosis and altering cell cycle progression. The biochemical characterization and understanding the localization and physiological function of these newly identified Cyts-*b*561 are awaited.

Based on primary sequence conservation and predicted structural features, four putative Cyt-*b*561 genes have been identified in *Arabidopsis thaliana* (Asard et al. 2001). Although there is limited homology between the predicted plant and animal sequences (approximately 30%), the presence of certain conserved residues and structural features support the similarity between the functions of these proteins. One of plant Cyts-*b*561 has already been over-expressed in yeast (*Saccharomyces serevisiae*) cells and the recombinant protein has been characterized to some extent (Griesen et al. 2004). The protein seems to be localized at the plant tonoplast (TCyt-*b*561) and encodes for an ASC-reducible Cyt-*b*561 with absorbance characteristics similar to that of CGCyts-*b*561. Presence of a Cyt-*b*561 in plant tonoplast was also obtained by characterizing an ASC-reducible *b*-type cytochrome in tonoplast-enriched bean membrane fraction (Preger et al. 2005).

Hardly any is known about the substrate specificity of Cyts-*b*561. The only study in this respect was done by Terland and Flatmark (1980) on highly-purified chromaffin granule ghosts from bovine adrenal medulla. It was shown that less than 1% of CGCyt-*b*561 was reduced by 0.1 mM NADH at pH 6.5 in 2 min. It is shown in this paper that the highly-purified recombinant mouse CGCyt-*b*561 can be reduced by millimolar concentrations of natural reductants, and dihydrolipoic acid (DHLA) seems to be as effective reductant as ASC.

Table 1. Effect of reductants on purified recombinant mouse CGCyt-*b*561 and its R72A mutant. For reference values, reduction at 25 mM of ASC is given as 100%; the 100% refers to 2.45 ± 0.75 nmol mg^{-1} and 1.20 ± 0.45 nmol mg^{-1} for CGCyt-*b*561 and its R72A mutant, respectively. All other measurements were at 2.5 mM of reducing agents. Values are means ± deviation from the means of two independent experiments.

Reducing agent potential	Percentage reduction		Midpoint redox
	CGCyt-b561	R72A-CGCyt-b561	$E_{m,7}$ (mV)
ASC (25 mM, ref.)	100	100	+320
ASC	75 ± 6	71 ± 1	+320 [(b)]
GSH	18 [(a)]	11 [(a)]	–230 [(c)]
DHLA	75 ± 5	69 [(a)]	–320 [(d)]
NADH	24 ± 5	0	–330 [(c)]
NADPH	0	0	–330 [(c)]
DTH	158 ± 6	122.2 ± 1	–480 [(e)]

[(a)] measured only once
[(b)] Washko et al. (1992), [(c)] Foyer and Noctor (2005), [(d)] Moini et al. (2002), [(e)] Mayhew (1978),

Materials and Methods

Molecular biology works

Yeast expression vector pESC-His containing the mouse CGCyt-*b*561 gene (Bérczi et al. 2005) was used as a template for PCR reactions to generate N and C-terminal His-tagged proteins. Primers were designed as given by Liu et al. (2005). Standard PCR methods were used to amplify the gene and amplified sequences were confirmed by DNA-sequencing at the University of Nebraska – Lincoln Genomic Core Research Facility.

For transformation, yeast cells (*Saccharomyces cerevisiae*, strain YPH499, ura3-52 lys-801^{amber} ade2-101^{ochre} trp1-Δ63 his3Δ200 leu2-Δ1) were grown in synthetic dextrose minimal medium (YPAD, Stratagene, La Jolla, CA) and transformation was performed according to manufactures instructions. Transformed lines were selected on SD dropout medium, lacking His (SD-His). For the induction of protein expression, overnight cultures were grown in SD-His, and transferred to 450 ml of synthetic galactose minimal medium (SG-His) containing 2% (w/v) galactose.

Yeast membrane preparation and stripping

Cells grown as 4 x 450 ml cultures were collected by low-speed centrifugation (5,000 g_{max} and room temperature for 10 min) when the OD_{600} reached a value of 0.8 to 1.0. Collected

Table 2. Kinetic constants obtained from data analysis of concentration dependent reduction by ASC and DHLA of CGCyt-*b*561 (Wild) and its R72A mutant (Fig. 1). Data analysis was performed with Origin5.0 software. Values are for the best fit to the experimental points with their uncertainty provided by Origin5.0.

CGCyt-b561	Reductant	"Apparent binding constant" for the High-affinity site (mM)	Low-affinity site (mM)
Wild	ASC	0.021 ± 0.006	1.50 ± 0.50
	DHLA	0.071 ± 0.020	-
R72A	ASC	-	1.48 ± 0.14
	DHLA	-	1.56 ± 0.10

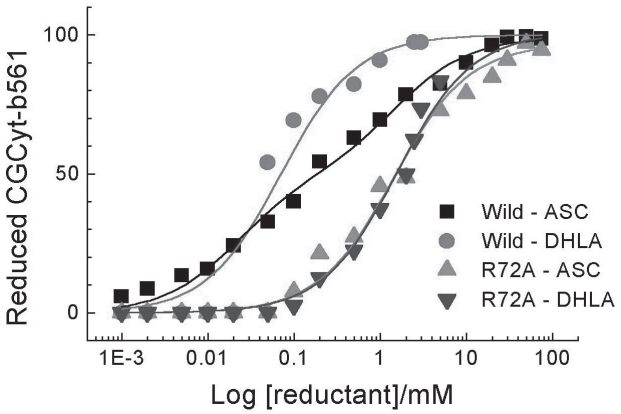

Figure 1. Concentration-dependent reduction by ASC or DHLA of highly-purified recombinant CGCyt-*b*561 (Wild) and its R72A mutant (R72A). For comparison, results are shown as percentage reduction of proteins. Experimental results are means from two independent experiments. Continuous lines are theoretical curves fitted by Origin5.0 software.

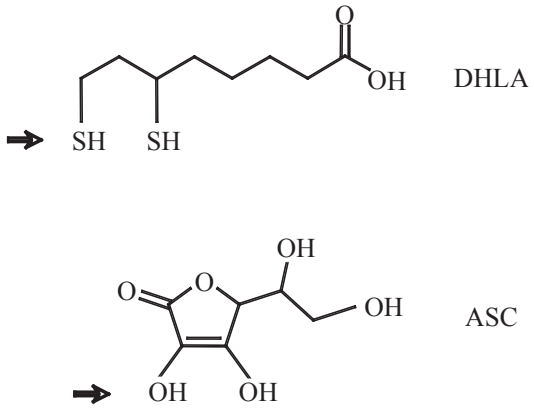

Figure 2. Chemical structure for ascorbic acid (ASC) and dihydrolipoic acid (DHLA) in their undissiociated forms. Arrows indicate the –SH and –OH groups discussed in the text.

cells were washed once with ice-cold homogenization buffer (50 mM MOPS-KOH, pH 7.0, 5 mM EDTA, 100 mM KCl, 100 mM sucrose) and pelleted as above. Microsomal membrane preparation and stripping of membranes were as described recently by Bérczi et al. (2005). Stripped membrane fractions were re-suspended in storage buffer (20 mM MES-Tris, pH 6.8, containing 2% (w/v) glycerol or 50 mM $NaH_2PO_4.H_2O$, pH 6.8, 2% (w/v) glycerol), and stored at –80°C until use.

Protein solubilization and purification by affinity chromatography

Membrane vesicles (about 80 mg protein) were resuspended in 50 mM $NaH_2PO_4.H_2O$, pH 6.8, 2% (w/v) glycerol, 1% (w/v) sucrose monolaurate (SML) at 2 mg ml^{-1} protein concentration. The mixture was incubated on a rocker at room temperature for a period of 60-90 min. Insoluble material was pelleted by high-speed centrifugation (Beckman Avanti centrifuge, JA-25.50 rotor) at 75,000 g_{max} and 4°C for 90 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 agarose resin (Ni-NTA His-bind Superflow, Novagen, Madison, WI) that had been pre-equilibrated with 50 mM $NaH_2PO_4.H_2O$, pH 7.8, containing 10% (w/v) glycerol, 1%(w/v) SML, 500 mM NaCl and 10 mM imidazole. The agarose resin was incubated with the solubilized proteins at room for 60 min. After incubation, the Ni-NTA agarose resin with bound proteins was collected in a 5-ml disposable column and washed thrice with 5 bed volumes of 50 mM $NaH_2PO_4.H_2O$, pH 7.8, containing 10% (w/v) glycerol, 0.1% (w/v) SML, 500 mM NaCl and 10 mM imidazole. His-tagged proteins were eluted with 6 bed volumes of 50 mM $NaH_2PO_4.H_2O$, pH 7.0, containing 10% (w/v) glycerol, 0.1%(w/v) SML, 150 mM NaCl and 250 mM imidazole. The eluate was concentrated by centrifugation using Centricon-YM100 centrifugal filter unit (Millipore, Bedford, MA) and desalted by using a PD-10 desalting column (Amersham Biosciences, Uppsala, Sweden). The desalted fraction was concentrated again and stored at –80°C until use.

Absorption spectroscopy

Absorption spectra were recorded in split beam mode (with buffer as reference) with an OLIS-updated SLM-Aminco DW2000 spectrophotometer (OLIS Co., Bogart, GA) with 1 nm slit-width, 0.5 nm s^{-1} scan rate, and under continuous stirring. First, Cyts were oxidized by addition of ferricyanide (0.5 mM, FeCN) and the fully oxidized spectra were recorded. Then, ASC-reduced (25 mM ASC) and ASC +dithionite-reduced (after addition of Na-dithionite crystals) spectra were obtained at room temperature. When improvement of the signal to noise ratio was needed, multiple scans were averaged. Cyt *b* amounts were calculated from the reduced-minus-oxidized difference spectra, obtained by subtracting the FeCN oxidized spectra from the ASC- or dithionite-reduced spectra, using a millimolar extinction coefficient of 30 mM^{-1} cm^{-1} (Tsubaki et al. 1997; Liu et al. 2005).

Results and Discussion

Although the major property of members of the Cyt-*b*561 protein family is their ASC-reducibility, hardly any work is known for studying the effect of other native and putative reducing agents present together with ASC in cells. Reason for this might be rooting in the facts that no pyridine nucleotide-binding site can be identified in any Cyt-*b*561 sequences and midpoint redox potentials for the most common reductants

in the cytosol are hundreds of millivolts lower than that for Cyts-*b*561.

Earlier studies on intact chromaffin secretory vesicles indicated that intra-granular ASC is regenerated by extra-granular ASC, however, other reducing agents, such as GSH, NADH and NADPH did not support this process (Dhariwal et al. 1991). Also, the ability of serotonin-containing dense granules of platelets, to accept electrons from NADH and NADHP were studied. Since serotonin-containing granule resembles that of chromaffin granules, the reduction was compared to CGCyt-*b*561 (Johnson and Scarpa 1981). In this study it was found that NADH could donate electrons to CGCyt-*b*561, but at a slower rate when compared to ASC. NADPH was unable to supply electrons. The ability of such physiologically relevant reducing agents to donate electrons to purified CGCyt-*b*561 has not yet been documented. Using the purified recombinant CGCyt-*b*561, the ability of NADH, NADPH, GSH, DHLA and dithionite (DTH) to reduce the CGCyt-*b*561 was tested.

Table 1 summarizes our results obtained when reduction of both FeCN-oxidixed recombinant mouse CGCyt-*b*561 and its R72A mutants were studied in the presence of 2.5 mM of both native and artificial reducing agents. It is clear that GSH as well as pyridine nucleotides are bad reducing agents for CGCyt-*b*561 but dithiol-containing reagents are capable of reducing both CGCyt-*b*561 and its R72A mutant. The physiological amounts of GSH in the cells vary from 0.5 mM to 10 mM (Wu et al. 2004) and that of NADH and NADPH vary from µM to mM range (Kirsch and de Groot 2001). Therefore the relevance of the observed reductions of CGCyt-*b*561 should be interpreted cautiously, as the values for NAD(P)H used (2.5 mM) are relatively high when compared to physiological levels. DHLA is present in mM concentrations in cells and is synthesized both in plants and animals (Packer et al. 1995; Moini et al. 2002). It is particularly interesting that (1) DHLA is as effective as ASC and (2) reduction by NADH is lost when R72 is replaced by A. This latter observation adds some emphasis to the predicted importance of R72 in the reduction mechanism of CGCyt-*b*561. There is not correlation between the reducing efficacy and midpoint redox potential of reductants tested.

It has been shown recently (Bérczi et al. 2005) that R72 seems to be responsible for the high-affinity ASC-binding of CGCyt-*b*561. It was concluded from (a) the lack of ASC-reducibility of R72A-CGCyt-b561 below 0.1 mM of [ASC] and (b) the kinetic parameters of data analysis. Concentration-dependent reduction of R72A-CGCyt-*b*561 by DHLA was very similar to that by ASC (Fig. 1). DHLA seems to have no low-affinity binding to CGCyt-*b*561. Data analysis of experiments (Table 2) revealed that reduction by ASC and DHLA might follow similar kinetics and reduction of R72A-CGCyt-*b*561 occurs only via low-affinity bindings of reductants. It is believed that reducing activity of DHLA resides in its –SH groups. Comparing (a) the chemical structure of ASC and DHLA (Fig. 2), (b) the midpoint redox potential values of ASC and DHLA (Table 1), and observing (c) the very similar reduction kinetics with these two reductants and the R72A mutant (Fig. 1), and (d) the slight difference between the high-affinity binding constants for ASC and DHLA, it might be probable that redox reactions at low reductant concentrations occur via specific interactions between the reductants and CGCyt-*b*561; for instance, R72 interacts with the two –SH groups on DHLA in a similar way as it does with the two –OH groups on ASC. Further studies are needed to clarifying the reduction mechanism by either ASC or DHLA.

In summary, it is shown that affinity-purified recombinant mouse CGCyt-*b*561 and its R72 mutant can be reduced not only by ASC (and DTH) but also by other native reductants. The reducing mechanism by DHLA shows similar properties to that by ASC. In both cases, an Arg residue in the predicted ASC-binding motive (R72) seems to play a key role in the high-affinity reduction of CGCyt-*b*561.

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