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An *Arabidopsis* cytochrome b561 with *trans*-membrane ferrireductase capability

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Abstract Ascorbate-reducible cytochromes b561 (Cyts-b561) are a class of intrinsic *trans*-membrane proteins. Tonoplast Cyt-b561 (TCytb), one of the four Cyt-b561 isoforms in *Arabidopsis* was localized to the tonoplast. We demonstrate here that the optical spectra, EPR spectra and redox potentials of recombinant TCytb are similar to those of the well characterized bovine chromaffin granule Cyt-b561. We provide evidence for the reduction of ferric-chelates by the reduced TCytb. It is also shown that TCytb is capable of *trans*-membrane electron transport from intracellular ascorbate to extracellular ferric-chelates in yeast cells.
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1. Introduction

Cytochromes b561 (Cyts-b561) are ascorbate (ASC)-reducible, intrinsic *trans*-membrane two-heme proteins [1–3], that have been identified in a great variety of organisms, including invertebrates, vertebrates, and plants [4,5]. All Cyts-b561 are predicted to consist of six *trans*-membrane helices, with the central four composing the "Cyt-b561 domain" [6]. This domain has four highly conserved His residues likely involved in the coordination of the two hemes [7,8]. The location of the four His residues and the two hemes on four consecutive *trans*-membrane helices of the CB domain distinguishes the Cyts-b561 from most other di-heme proteins [8].

The only extensively studied Cyt-b561 protein is the one in chromaffin granule membranes of neuroendocrine tissues, the chromaffin granule Cyt-b561 (CGCytb). This protein is generally believed to catalyze the reduction of intra-vesicular monodehydroascorbate at the expense of cytoplasmic ASC [1,2,9–11]. This electron-transfer reaction replenishes ASC levels inside the granules, supporting the activity of intra-vesicular monooxygenases [12,13]. Both the native and the recombinant forms of the bovine CGCytb have been purified [14,15]. However, crystallization and 3-D structure determination of the protein has not yet been achieved.

Recently other mammalian Cyts-b561 were identified, respectively located in the duodenal brush border membrane, duodenal Cyt-b561 (Dcytb) [16,17], and macrophage lysosomes, lysosomal Cyt-b561 (LCytb) [18]. DCytb was also found in human erythrocyte membranes where it may function as a monodehydroascorbate reductase [19]. Three mammalian Cyts-b561, DCytb, CGCytb and LCytb, showed ASC-dependent *trans*-membrane ferrireductase activity when expressed in yeast cells [20]. The observation that Cyts-b561 may be involved in iron metabolism has opened new perspectives for their physiological function.

Arabidopsis and other plants contain four putative Cyts-b561 [3]. However, little is known about their localization and physiological function. One isoform (formerly called At-Cytb561-1 [3] and CYBASC1 [20]) is localized on the tonoplast [21–23] and is named tonoplast Cyt-b561 (TCytb). It has been speculated that this protein could be involved in iron metabolism and ASC regeneration in plant cells. TCytb has been partially purified from bean [23], and has been expressed in yeast cells [21]. Here we describe the biophysical properties of recombinant TCytb, and characterize its ferrireductase activity.

2. Materials and methods

2.1. Yeast strains and growth media

Yeast cells (*Saccharomyces cerevisiae*, strain YPH499, ura3-52 lys2-801[amber] ade2-101[ochre] trp1-Δ63 his3-Δ200 leu2-Δ1) were grown in synthetic dextrose (SD) minimal medium (Stratagene, La Jolla, CA, USA). Yeast strains, S288C (MATα ura3-52 leu2-1) and S288C Δfre1Δ fre2 (MATα fre1Δ::URA3 fre2Δ::HISG leu2-1), are kind gifts from Dr. Jerry Kaplan (University of Utah). The cells were grown in medium containing either yeast extract-peptone-dextrose (YPD), or SD, or synthetic Gal (SG) minimal media.

2.2. Plasmid constructs, gene cloning and protein expression

Standard PCR methods were used to amplify the *TCytb* gene from *Arabidopsis* mixed tissue total RNA, and *Fre1* from yeast genomic DNA [20,21]. Both genes were cloned into the pESC-Leu or pESC-His vector (Stratagene) and transformed into the Δfre1Δfre2 or YPH499 yeast cells. Transformed lines were selected on SD drop-out medium lacking His (SD-His) or Leu (SD-Leu). Protein expression was induced by transfer of overnight cultures to 500 ml of SG minimal medium containing 2% (w/v) Gal and 1 mg ml^{-1} sodium ASC.

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Abbreviations: ASC, ascorbate; FeCN, ferricyanide; Gal, galactose; GL, L-galactono-γ-lactone; MMF, microsomal membrane fraction; Cyt-b561, cytochrome b561; CGCytb, chromaffin granule Cyt-b561; DCytb, duodenal Cyt-b561; LCytb, lysosomal Cyt-b561; TCytb, tonoplast Cyt-b561

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2.3. Yeast membrane preparation, stripping, solubilization and protein purification

Cells from 1800 ml cultures were collected at density of $OD_{600} = 0.9 \pm 0.1$. Preparation of microsomal membrane fraction (MMF), stripping of membrane vesicles and partial purification of TCytb were as given in [24]. Stripped membrane vesicles were stored in MES-Tris buffer (20 mM MES, 2% (w/v) glycerol, pH 7 with Tris salt) at −80 °C until use. Solubilization with sucrose monolaurate (SML, Dojindo Laboratories, Japan) and protein purification on a Q-Sepharose column (Amersham Biosciences, Uppsala, Sweden) were performed as described before [24].

Protein determination and Western blots were performed as described [20], using BSA and TCytb-specific antibodies [21].

2.4. Spectroscopy

Absorption spectra of TCytb in membrane vesicles were recorded in dual-wavelength mode (between 500 and 600 nm, reference at 601 nm; OLIS-Aminco DW2000 spectrophotometer, Bogart, GA) with 1 nm slit-width, 0.5 nm s^{-1} scan rate, in the presence of 100 mM of KCl and 0.025% (w/v) of Triton X-110, and under continuous stirring. Absorption spectra of TCytb in detergent micelles were recorded in split-beam mode (between 360 and 660 nm, reference at 666 nm) with 1 nm slit-width, 0.5 nm s^{-1} scan rate, and under continuous stirring. Spectra were taken at room temperature and multiple scans were averaged to improve the signal to noise ratio. For calculations, reduced minus oxidized difference spectra and a millimolar extinction coefficient of 30 mM^{-1} cm^{-1} at 561 nm [5,15] was used.

EPR spectroscopy was performed with the fully oxidized protein in detergent micelles in phosphate buffer as described [24].

2.5. Optical redox titration

Optical redox titration was carried out with SML-solubilized proteins in MES-Tris buffer containing 0.1% (w/v) SML and 10% (w/v) glycerol under anaerobic conditions [24]. Spectra were recorded between 500 and 600 nm in split-beam mode and the reduced minus oxidized difference spectra were used for calculations.

2.6. Ferrireductase assays

TCytb in stripped microsomal membranes was reduced by 10 mM ASC. ASC was removed by sedimenting at 75000 g_{max} for 30 min and resuspending pellets in MES-Tris buffer. Spectra were recorded in dual wavelength mode as detailed above. Ferricyanide (FeCN), Fe(III)-EDTA, and Fe(III)-citrate were added from 100-fold stock solutions freshly prepared in MES-Tris buffer.

Cell surface ferrireductase assays with yeast cells expressing TCytb were performed exactly as described previously [20].

3. Results and discussion

3.1. Biophysical properties of TCyb

MMF from yeast cells expressing TCytb shows an ASC-reducible b-type Cyt spectrum with α-band absorbance maximum near 561 nm and a shoulder near 557 nm (Fig. 1). No other b-type cytochromes were detected in the MMF from yeast cells transformed with the empty vectors. The small amount of contaminating cytochrome *c* (small peak at 550 nm) was removed by stripping of membrane vesicles before solubilization. A typical protein yield was 40 mg protein with 30 nmol TCytb in the stripped MMF from 1800 ml of culture (at $OD_{600} = 0.9 \pm 0.1$). Therefore, the yeast expression system is useful for large-scale expression of TCytb. After solubilization, chromatography, and protein concentration steps, TCytb recovery was about 30%.

Three biophysical properties are widely used for the characterization of two-heme proteins; the UV–Vis spectra, the low-temperature EPR spectra, and the heme redox potential values. The optical absorption spectra of the fully-oxidized and ASC- and dithionite-reduced TCytb (Fig. 2A) show characteristic bands that are identical to those obtained for CGCytb under similar conditions [15,24]. The EPR spectrum of the partially purified TCytb at 5, 10, and 15 K (Fig. 2B) reveals the presence of two-hemes (g_z values of 3.14 and 3.69) with a similar micro-environment as that observed for CGCytb in its fully oxidized form (g_z values of 3.14 and 3.70 [14], 3.14 and 3.72 [15], and 3.27 and 3.71 [24]). Finally, the optical redox titration of TCytb in detergent micelles at room temperature reveals the presence of two one-electron redox centers with E'_0 values of 57 ± 20 mV and 165 ± 26 mV ($n = 3$) (Fig. 2C). These characteristics have not previously been obtained for TCytb and together clearly demonstrate that this protein belongs to the ASC-reducible two-heme-containing Cyt-b561 protein family.

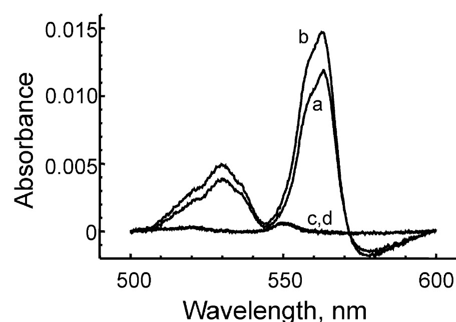

Fig. 1. Reduced minus oxidized difference spectra of MMFs from yeast cells expressing the pESC-His vector (spectra c and d) or TCytb (spectra a and b). The FeCN-oxidized proteins were reduced by 20 mM sodium ASC (spectra a and c) or by 2 mM sodium dithionite (spectra b and d). Spectra were obtained at room temperature and 1 mg ml^{-1} protein concentration. Similar results were obtained with pESC-Leu vector (data not shown). The minor peaks at 550 nm in spectra c and d indicate the presence of contaminating Cyt c probably trapped in sealed vesicles.

3.2. Ferric-chelate reduction by TCytb

FeCN is generally used to oxidize reduced Cyts. One member of the Cyt-b561 family, DCytb, has been suggested to participate in ferric-chelate reduction and iron acquisition [16,17]. In addition to FeCN, we tested the reduction of Fe(III)-EDTA and Fe(III)-citrate by reduced TCytb (Fig. 3). Although the redox potential for Fe(III)-EDTA and Fe(III)-citrate is significantly lower (+130 mV and +200 mV [25]) than that for FeCN (+360 mV [25]), all three compounds were able to accept electrons from reduced TCytb. This result is consistent with a potential role for TCytb in ferric-chelate reduction.

To test whether the recombinant TCytb could function as a ferrireductase in vivo, plasma membrane ferrireductase activities were measured using Δfre1Δfre2 yeast cells expressing TCytb. Fre1 and Fre2 are yeast ferrireductases that account for almost all of the plasma membrane ferrireductase activity [26]. ASC is commonly accepted as the physiological electron donor for the Cyts-b561. However, yeast synthesizes erythroascorbate, not ASC [27]. It is not known whether erythroascorbate can reduce Cyts-b561. However, yeast cells can synthesize ASC when given the precursor L-galactono-γ-lactone (GL). Therefore, ferrireductase measurements were carried out with cells grown in the absence and presence of GL. As expected, little ferrireductase activity was seen in the Δfre1Δfre2 mutant transformed with the empty vector (Fig. 4). Transformation with *Fre1* resulted in high FeCN reductase activity, which

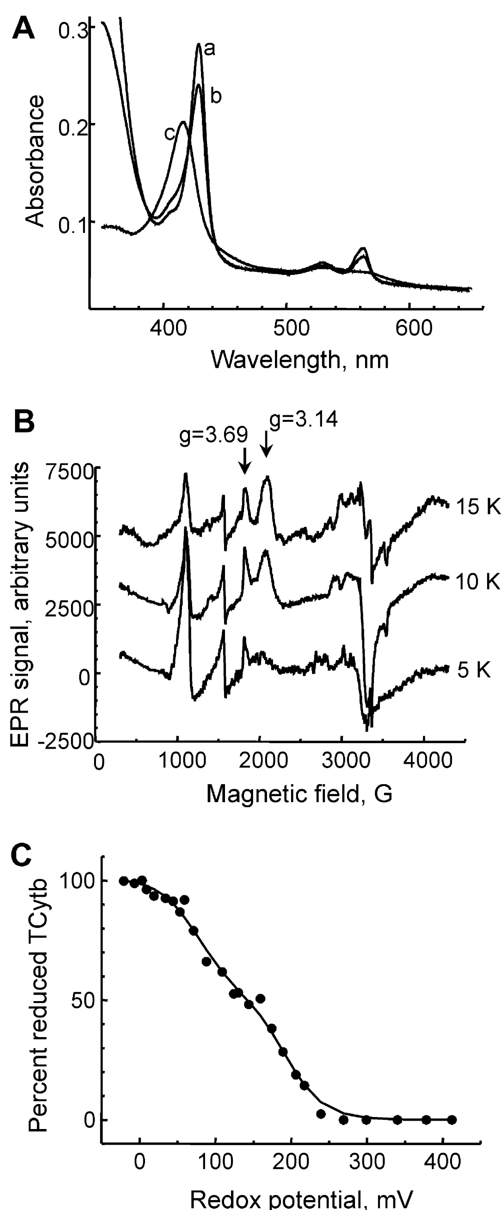

Fig. 2. Absorption spectra (A), EPR spectra (B), and redox titration (C) of partially purified recombinant TCytb. (A) TCytb was oxidized by FeCN (0.2 mM, spectrum a), reduced by ASC (20 mM, spectrum b) or by dithionite (2 mM, spectrum c) and spectra were recorded at room temperature. The presented curves are averages of four scans. (B) The EPR signals were recorded at 5, 10, and 15 K, 10 mW microwave power, 9.38 GHz microwave frequency, 100 kHz modulation frequency, and 10 G modulation amplitude. The presented curve is the averages of four scans. (C) Redox titration was performed under anaerobic conditions at pH 7.0. Dithionite was added stepwise to the fully-oxidized protein and, after stabilization of the potential in the optical cuvette, the spectrum was recorded between 500 nm and 600 nm. For calculations, reduced minus fully-oxidized difference spectra were taken and integrated between 500 and 600 nm. The difference between the maximal and minimal value was taken as reference (100%) value and titration is shown as percentage reduced TCytb vs. bulk potential in the assay. Experimental points are from one titration. The curve was calculated using the fitted redox potential values.

was independent of the presence of GL. Expression of TCytb in the Δfre1Δfre2 mutant cells also resulted in high FeCN

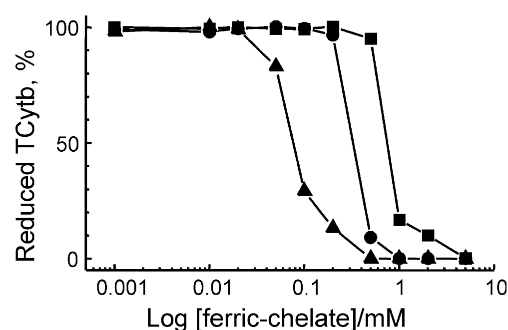

Fig. 3. Oxidation of reduced TCytb in stripped MMFs by FeCN (circles), Fe(III)-EDTA (squares), and Fe(III)-citrate (triangles). One hundred percent value refers to 1.45 nmol ml^{-1} reduced TCytb in the optical cuvette. Results are from one series of experiments.

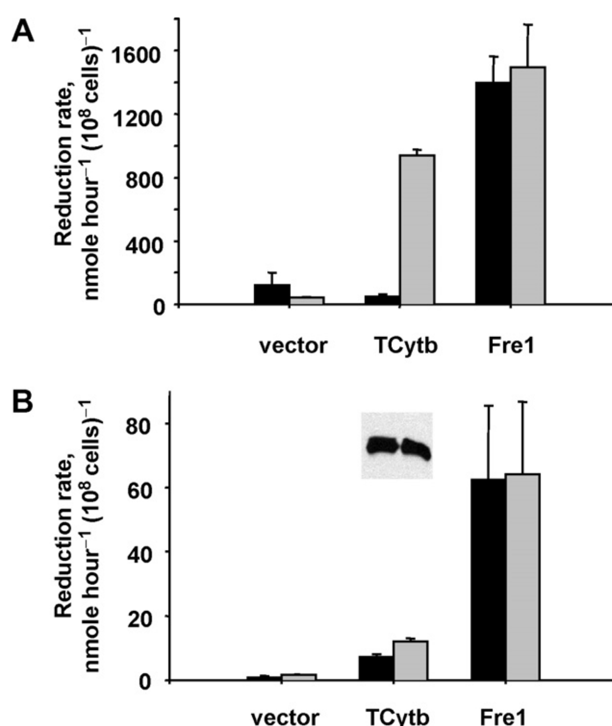

Fig. 4. Reduction of FeCN (A) and Fe(III)-EDTA (B) by Δfre1Δfre2 yeast cells expressing either the empty vector, or TCytb, or Fre1. Ferric-chelate reduction was determined in the absence (black columns) or presence (grey columns) of GL in the growth medium. Western blot analysis proves that expression of TCytb was independent of the presence of CG in the growth medium (insert). Results are averages ± S.D. of three independent series of experiments.

reductase activities, and these activities were almost completely dependent on GL addition. GL did not affect protein expression levels as demonstrated by Western blots (Fig. 4, insert).

With Fe(III)-EDTA as a substrate, the ferrireductase activity of the Δfre1Δfre2 mutant cells transformed with *TCytb* was again significantly higher than that of vector-only transformed cells (Fig. 4B). Because we have no means to quantitatively compare TCytb and Fre1 expression levels, it is difficult to compare the activity of these proteins. Overall, the ferrireductase activities with Fe(III)-EDTA were about 20- to 35-fold and 60- to 75-fold lower than that with FeCN as substrate for

cells expressing Fre1 or TCytb, respectively. Fe(III)-EDTA reductase activity of cells expressing TCytb increased with addition of GL to the growth medium, however, the effect was less dramatic than in the FeCN reduction. These ferrireductase results are in full agreement with those obtained with recombinant mammalian Cyt-b561 proteins under similar conditions [20].

In summary, our results demonstrate that the biophysical properties of the recombinant plant TCytb, expressed in yeast, are similar to those of the mammalian CGCytb. Moreover, TCytb catalyzes ASC-dependent *trans*-membrane ferric-chelate reduction in intact yeast cells.

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