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Heterologous production and characterisation of two distinct dihaem-containing membrane integral cytochrome b_{561} enzymes from *Arabidopsis thaliana* in *Pichia pastoris* and *Escherichia coli* cells

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ABSTRACT

Cytochrome (cyt) b_{561} proteins are dihaem-containing membrane proteins, belonging to the CYBASC (cytochrome-b_{561}-ascorbate-reducible) family, and are proposed to be involved in ascorbate recycling and/or the facilitation of iron absorption. Here, we present the heterologous production of two cyt b_{561} paralogs from *Arabidopsis thaliana* (Acytb_{561}-A, Acytb_{561}-B) in *Escherichia coli* and *Pichia pastoris*, their purification, and initial characterisation. Spectra indicated that Acytb_{561}-A resembles the best characterised member of the CYBASC family, the cytochrome b_{561} from adrenomedullary chromaffin vesicles, and that Acytb_{561}-B is atypical compared to other CYBASC proteins. Haem oxidation–reduction midpoint potential (E_M) values were found to be fully consistent with ascorbate oxidation activities and Fe^{3+}-chelates reductase activities. The ascorbate dependent reduction and protein stability of both paralogs were found to be sensitive to alkaline pH values as reported for the cytochrome b_{561} from chromaffin vesicles. For both paralogs, ascorbate-dependent reduction was inhibited and the low-potential haem E_M values were affected significantly by incubation with diethyl pyrocarbonate (DEPC) in the absence of ascorbate. Modification with DEPC in the presence of ascorbate left the haem E_M values unaltered compared to the unmodified proteins. However, ascorbate reduction was inhibited. We concluded that the ascorbate-binding site is located near the low-potential haem with the Fe^{3+}-chelates reduction-site close to the high-potential haem. Furthermore, inhibition of ascorbate oxidation by DEPC treatment occurs not only by lowering the haem E_M values but also by an additional modification affecting ascorbate binding and/or electron transfer. Analytical gel filtration experiments suggest that both cyt b_{561} paralogs exist as homodimers.

1. Introduction

Cytochrome (cyt) b_{561} proteins are di-haem containing membrane proteins that can shuttle one electron across the lipid bilayer and belong to the eukaryotic redox protein family CYBASC [1,2]. The function of the CYBASC proteins has mostly been correlated with ascorbate recycling [3–7] and/or facilitation of iron absorption [8–12]. In animals and plants, several CYBASC paralogs were shown to be present [1]. Multiple alignment of amino acid sequences of various CYBASC members has suggested that they all possess a conserved three-dimensional (3-D)

Abbreviations: A_x, absorption at a wavelength of x nm; BCA, bicinchoninic acid; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; BSA, bovine serum albumin; CV, column volumes; CYBASC, cytochrome b_{561} ascorbate reducible family; cyt b_{561}, cytochrome b_{561}; Da, Dalton; $\Delta E_{M,bH}$, shift in $E_{M,bH}$; $\Delta E_{M,bL}$, shift in $E_{M,bL}$; DEPC, diethyl pyrocarbonate; DDM, n-dodecyl-β-D-maltoside; DMSO, Dimethyl sulfoxide; DTT, Dithiothreitol E_M, oxidation–reduction midpoint potential; $E_{M7.2}$, E_M value at pH 7.2; $E_{M,bH}$, E_M of the high-potential haem; $E_{M,bL}$, E_M of the low-potential haem; ESI, Electrospray ionisation; FOS-12, Fos-choline 12, H_{10}, deca-histidine affinity tag; IEF, isoelectric focusing; IMAC, immobilised metal affinity chromatography; IPTG, Isopropyl β-D-1-thiogalactopyranoside; LB, Luria-Bertani; MDHA, mono-dehydroascorbate; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; MS, Mass spectrometry; MW, molecular weight; M9, Salts Minimal Media; NZYM, NZ amine; NaCl, bacto-yeast extract and magnesium sulphate; OD_x, optical density at a wavelength of x nm; ORF, open reading frame; RCA, relative catalytic activity; S_{II}, streptavidin affinity tag II; *S. c.*, *Saccharomyces cerevisiae*; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TMPD, N,N,N′,N′,tetramethyl-p-phenylenediamine dihydrochloride; YNB, Yeast Nitrogen Bases; *Zm*, *Zea mays*

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structure with six highly hydrophobic areas predicted to form membrane-spanning α-helices, four conserved His residues likely to be involved in the coordination of two haem *b* centres, and the putative ascorbate and mono-dehydroascorbate (MDHA) binding motifs [1,13–15] (Supplementary Fig. S1). While investigation of animal CYBASC members has proceeded rapidly in the recent years, little has been presented on plant CYBASC and the physiological function in plants of this redox family is still unknown. The first putative cyt b_{561} purified from the plasma membrane of bean hypocotyls (*P*cytb_{561}-I), was long believed to be the plant counterpart of the bovine chromaffin granule cyt b_{561} [16,17]. However, a recent extensive biochemical characterisation of *P*cytb_{561}-I has shown that this cytochrome is not a CYBASC member [18]. To date, the purified plant CYBASC proteins are the *A*cytb_{561}-A from *Arabidopsis thaliana* [8,19,20], *C*cytb_{561}-A from wild water melon [21], a cyt b_{561} from bean (*P*cytb_{561}-II) [22] and one protein from *Zea mays* (Zmcytb_{561}) [23]. Although all these proteins displayed typical CYBASC features, they have been found in different subcellular compartments and may perform different physiological functions. For instance, *A*cytb_{561}-A and the *P*cytb_{561}-II from bean are localised in the tonoplast and both are proposed to be involved in iron homeostasis [8,18,19], while the *C*cytb_{561}-A has been identified in the plasma membrane and is proposed to be involved in energy dissipation [21].

The *A. thaliana* genome appears to contain four cytb_{561} genes [1,24,25]. Based on microarray data and ESTs (expressed sequence tags), all of them are expressed in vivo (RNA level). On the protein level, the two genes *artb561-a* and *artb561-b* were shown to be expressed [13,19,26]. In addition, expression of cytb_{561} proteins was shown in *Rhaphanus sativus* [26].

Currently, no experimentally determined three-dimensional atomic model of any CYBASC member has been reported. Therefore, studies that enable production of CYBASC members are essential to future structural studies of representatives of this superfamily. Here we present the heterologous production in *Escherichia coli* and *Pichia pastoris* strains and the initial biochemical characterisation of *A*cytb_{561}-A and *A*cytb_{561}-B, two distinct members of the CYBASC family. The absorbance spectra of reduced *A*cytb_{561}-B differed from those of chromaffin granule cyt b_{561} and *A*cytb_{561}-A. However, biochemical characterisation of the purified *A*cytb_{561}-A and *A*cytb_{561}-B confirmed that both proteins are CYBASC members.

2. Material and methods

2.1. Generation of expression constructs

In order to introduce the coding sequence for the streptavidin affinity tag II (S_{II}) and deca-histidine (H_{10}) affinity tag into the pPIC3.K expression vector (Invitrogen), two complementary ssDNA oligonucleotides were synthesised (around 90 base pairs each), annealed and ligated to the pPIC3.5K plasmid previously digested with *Bam*HI/*Not*I restriction endonucleases (BioLab). The *artb561-b* ORF was amplified by PCR using the ′CGG AAT TCG CGG TTC CGG TGC TG′ and′CGA CCC TAG GTT GTG TGA GAA CTT GAT CC′ primers. Fragments were digested with the *Avr*II and *Eco*RI restriction endonucleases (BioLab) and cloned into the *Avr*II and *Eco*RI restriction sites of the pPIC3.5K-S_{II}/H_{10} derivative. In order to insert the ORFs encoding for the cyt b_{561} proteins into the pBAD expression vector derivatives, *artb561-a* was amplified using the ′GTA GCT GAG ATC TTC GCT GTC CGG ATA AAC′ and ′GTA GCT GGA ATT CGC TAT AGC AGA ATA ACT′ primers while the *artb561-b* was amplified with ′GTA GCT GAG ATC TTC GCG GTT CCG GTG CTG′ and ′GTA GCT GGA ATT CCG TTG TGT GAG AAC TTG′ primers. The DNA fragments so obtained and the pBAD expression vector derivatives were digested with *Bgl*II and *Eco*RI restriction endonucleases (BioLab) and ligated together. In all cases, the correct insertion of the ORFs into the expression vector derivatives was verified by colony PCR, restriction analysis and DNA sequencing (SeqLab GmbH, Göttingen).

2.2. Protein production in P. pastoris and membrane preparation

Protein production in *P. pastoris* was performed essentially as described previously for other membrane proteins by Weiss and coworkers [27] and is described in detail in the supplementary material. Normally from 12 L of cell culture, 100–120 g wet weight of cell pellet were obtained. The membrane preparation steps are described in detail in the supplementary material.

2.3. Creation of a methionine-auxotrophic E. coli strain

Methionine-auxotrophic strains of *E. coli* Top10-pRARE (F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) (Invitrogen) were obtained after mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and negative selection using ampicillin [28]. The procedure is described in detail in the supplementary material.

2.4. Protein production in E. coli and membrane preparation

Protein production in *E. coli* was performed essentially as described previously for other membrane proteins by Surade and coworkers [29] and is described in detail in the supplementary material. Normally from 24 L cell culture, 40–60 g wet weight of cell pellet were obtained. The membrane preparation steps are described in detail in the supplementary material.

2.5. Protein quantification and purification (by IMAC)

Protein concentration was determined using the BCA Kit (Pierce) protocol [30] using bovine serum albumin (BSA) as a standard. Purified proteins were concentrated by pressure dialysis using 14 mL concentrators with a cut-off of 30 kDa (Amicon). Purification was performed using cell membranes obtained from, respectively, 2 L of *P. pastoris* or 24 L of *E. coli* cell culture. All chromatographic steps were performed at 4 °C with ice-cold buffers and at a flow rate of 0.5 ml/min on an ÄKTA Purifier system (GE Healthcare). Cell membranes at 5 mg/mL total protein concentration were solubilised in the dark with constant stirring at 4 °C for 2 h in 50 mM Na-phosphate pH 7.2, 350 mM NaCl, 10% glycerol (buffer A), with the addition of 1% Fos-choline 12 (Fos12), 25 mM ascorbate, 1 M betaine and 0.5 mM histidine. Insoluble material was separated from solubilised proteins by ultracentrifugation at 100,000×*g* for 1 h at 4 °C. The supernatant was filtered through a 0.2 μm filter and loaded onto a HisTrap™ column (GE Healthcare) pre-equilibrated in 50 mM Na-phosphate pH 7.2, 350 mM NaCl, 10% glycerol, 0.1% Fos12 (buffer A1). The column was washed with 10 column volumes (CV) of buffer A1. In order to exchange the detergent from Fos12 to n-dodecyl-β-D-maltoside (DDM), the column was equilibrated with 5 CV of buffer A2 (50 mM Na-phosphate pH 7.2, 350 mM NaCl, 10% glycerol, 0.1% DDM). This was followed by a second wash in buffer A2 mixed with 2.5% buffer B (50 mM Na-phosphate pH 7.2, 350 mM NaCl, 10% glycerol, 250 mM histidine, 15 mM ascorbate, 5 mM betaine). The recombinant cyt b_{561} was eluted from the column in 100% buffer B.

2.6. Analytical gel filtration

The IMAC-purified cyt b_{561} proteins were analysed using a Superdex 200 PV 3.2/30 gel filtration column on a Smart chromatographic station (GE Healthcare). The Superdex 200 column was equilibrated at room temperature with at least 2.5 bed volumes of buffer A2 (50 mM Na-phosphate pH 7.2, 350 mM NaCl, 10% glycerol, 0.1% DDM). 50 μL of 1 mg/ml purified recombinant cyt b_{561} proteins