Characterization of an ascorbate-reducible cytochrome b561 by site-directed mutagenesis

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ABSTRACT

Ascorbate(ASC)-reducible cytochrome b561 (Cyt-b561) proteins are present in both plants and animals and create a well-distinguished protein family amongst the two-heme containing b-type cytochromes. One isoform of the Cyt-b561 identified by genomic analysis of Arabidopsis thaliana has been localized in the tonoplast. We have expressed the tonoplast-localized Cyt-b561 (TCyt-b561) in yeast (Saccharomyces cerevisiae) cells and shown that the biophysical properties of the recombinant TCGCyts-b561 is very similar to those of the chromaffin granule Cyt-b561 (CGCyts-b561). Mutation of 4 well-conserved histidine residues (H50, H83, H117, H156) resulted in different expression levels and revealed the importance of these 4 His residues in heme binding and protein expression. Modification of the protein by FLAG-tag or His-tag resulted in different degrees of reduced expression levels. When all lysine residues (K70, K76, K79, K80, and K159) in the vicinity of the putative ASC-binding motive were one-by-one replaced by alanine, no major changes in the expression levels were observed. Except in case of the K80A mutant, where the low-affinity ASC-binding constant increased significantly, there were no significant changes in either kinetic parameter characterizing the bi-phase ASC-dependent reduction of TCytb-b561. These observations are discussed in comparison to properties of the recombinant CGCyts-b561.

KEY WORDS

ASC reduction site-directed mutagenesis cytochrome b561 essential histidine residues FLAG-tag His-tag lysine residues

Ascorbate(ASC)-reducible cytochromes b561 (Cyts-b561) are a newly described class of intrinsic trans-membrane proteins with two hemes (Wakefield et al. 1986a,b; Asard et al. 2001). Cyts-b561 have been identified in a great variety of organisms, including invertebrates, vertebrates, and plants as well (Verelst and Asard, 2003; Tsuchaki et al. 2005). All Cyts-b561 are predicted to consist of six trans-membrane helices, the central four of them being identified as “Cyt-b561 (CB) domain”, a structural domain associated with other protein domains in other protein families (Pointing 2001). The CB domain has 4 highly conserved His residues likely involved in coordination of the two hemes (Okuyama et al. 1998). The special location of the four His residues on and two hemes in-between the 4 consecutive trans-membrane helices are one of the common structural characteristics of the Cyt-b561 proteins (Bashotovyy et al. 2003).

The only well characterized Cyt-b561 protein is the one in chromaffin granule membranes of neuroendocrine tissues, the CGCyts-b561. It is believed to catalyze the reduction of intravesicular monodehydroascorbate at the expense of cytoplasmic ASC (Njus et al. 1983; Wakefield et al. 1986a,b; Dhariwal et al. 1991). This electron-transfer reaction replenishes ASC levels inside the granules, supporting the activity of intravesicular monoxygenases such as dopamine β-hydroxylase and peptidyl glycine α-amidating monoxygenase (Kent and Fleming 1987; Seike et al. 2003). Both the natural and the recombinant form of the bovine CGCyts-b561 has been purified to homogeneity (Tsubaki et al. 1997; Liu et al. 2005), however, crystallization and 3-D structure determination of the protein was not yet been achieved.

Four putative Cyt-b561 isoforms have been identified first in Arabidopsis thaliana (AtCyt-b561-1 through AtCyt-b561-4) and then in other plants (Asard et al. 2001), however, very little is known about their cellular and/or organ localization and function. One isoform, the AtCyt-b561-1 (formerly called CYBASC1 in Griesen et al. 2004), has recently been identified in the tonoplast (Griesen et al. 2004; Shimaoka et al. 2004; Preger et al. 2005), and there is also a speculation of its participation in the iron metabolism of as well as ASC regeneration in the plant cells. Although the tonoplast-localized protein has already been expressed in yeast cells (rAtCyt-b561-1; Griesen et al. 2004), the recombinant protein has not yet been purified and characterized. A tonoplast-localized protein has been purified only partially from beans (Preger et al. 2005) but hardly been characterized yet.

In this paper we show that (1) 4 well-conserved His residues of the recombinant tonoplast-localized AtCyt-b561-1 (hereafter called TCyt-b561) are essential for heme co-ordinations, (2) FLAG- or His-tag influences the expression levels, and (3) replacing of K80 by Ala increases significantly the low-affinity ASC-binding constant but no other K-to-A replacement influences the ASC reducibility or the kinetics of ASC reduction.

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Materials and Methods

Plasmid construct and yeast transformation

Standard PCR methods were used to amplify the gene encoding AtCyt-b561-1 from Arabidopsis mixed tissue total RNA. Primers were designed to include EcoRI and SpeI sites for cloning into the pESC-His expression vector (Stratagene, La Jolla, CA, USA), downstream of the GAL10 Gal-inducible promoter. Sequences were confirmed by DNA sequencing at the University of Nebraska – Lincoln Genomic Core Research Facility.

For the tagged proteins, yeast expression vector pESC-His containing the AtCyt-b561-1 gene (Griesen et al. 2004) was used as a template for PCR reactions to generate N- and C-terminal His-tagged proteins as well as C-terminal FLAG-tagged proteins. Primers were designed as given by Liu et al. (2005) and Griesen et al. (2004), respectively. 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 transformations, yeast cells (Saccharomyces cerevisiae, strain YPH499, ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2Δ1) were grown in synthetic dextrose (SD) minimal medium (Stratagene, La Jolla, CA, USA) and transformation was performed according to manufacturers instructions. Transformed lines were selected on SD drop-out medium lacking His (SD-His). For induction of protein expression, overnight cultures were grown in SD-His and were transferred to 500 ml of synthetic galactose (SG) minimal medium containing 2% (w/v) galactose and 1 mg/ml sodium ASC.

Antibody production and purification

Antibodies were generated against the 21 amino acid C-terminal polypeptide ([Cys]-SPSPSPSVNDDDSVDFSYSI) of predicted AtCyt-b561-1 as described before (Griesen et al. 2004). Affinity purified antibodies were used in immunodetections.

Yeast membrane preparation and membrane stripping

Cells from 4 x 450 ml cultures were collected by low-speed centrifugation when the OD_600 reached a value of 0.9 ± 0.1. Preparation of the microsomal membrane fraction, stripping of membrane vesicles and protein determination were as given by Bérczi et al. (2005). Stripped membrane vesicles were stored in MES-Tris buffer (20 mM MES, 1% (w/v) glycerol, pH adjusted to 7 by addition of Tris salt) at –80ºC until use.

Spectroscopy

Absorption spectra of rTCyt-b561 in the microsomal membrane vesicles were recorded in dual-wavelength mode (between 500 and 600 nm and reference at 601 nm; OLIS-
updated SLM-Aminco DW2000 spectrophotometer, Bogart, GA) with 1 nm slit-width, 0.5 nm s⁻¹ scan rate, and under continuous stirring. Spectra were taken at room temperature in 20 mM MES-Tris buffer, pH 7, 1% (w/v) glycerol, 0.025% (w/v) Triton X-100, 100 mM KCl and in the presence or absence of 0.05 mM ferricyanide or 25 mM ASC or 2.5 mM dithionite. Multiple scans were averaged if improvement of the signal to noise ratio was needed. For calculations, reduced minus oxidized difference spectra and a millimolar extinction coefficient of 30 mM⁻¹ cm⁻¹ at 561 nm (Tsubaki et al. 1997; Liu et al. 2005) were used. Data analysis and curve fitting was done by using the SPSERV and Origin5.0 softwares. Percent- age ASC-reducibility is the ratio of reduced dithionite to dithionite reduced TCyt-b561 multiplied by 100. Data presented on Figs. 1 and 3 are means with standard deviations of at least three independent series of experiments.

**Gel electrophoresis and Western blotting**

All steps were as detailed by Griesen et al. (2004). Shortly, samples were not heated or boiled prior to loading on the gels, because this caused the proteins recognized by the TCyt-b561 antibodies to aggregate, preventing them from penetrating into the gel. Proteins were resolved by SDS-PAGE electrophoresis, using 12% acrylamide gels and transferred onto polyvinylidene difluoride membranes (PVDF, Bio-Rad, Hercules, CA) with a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) in 12 mM Tris, 96 mM glycine, 20% (v/v) methanol. Protein-antibody complexes were detected by horseradish peroxidase-conjugated secondary antibodies (ECL detection kit, Amersham-Pharmacia Biotech, Piscataway, NJ).
Result and Discussion

His mutants and tagged proteins

It has been shown with recombinant mouse CGCyt-b561 that the H52-H120 and the H86-H159 pairs of His residues take place in coordination of the two hemes (Bérczi et al. 2005). While the mutation from H to A at positions of H52 and H120 resulted in a loss of protein expression, H-to-A replacement at positions of H86 and H159 resulted only in significant reduction of protein expression. In full agreement with those results, the H50-H117 and the H83-H156 pairs of His residues seem to coordinate two hemes in TCyT-b561 (Fig. 1). While the H-to-A replacement at positions of H50 and H117 resulted in total loss of detectability of TCyT-b561, the H-to-A mutation at positions of H83 and H156 resulted only in reduced specific content of TCyT-b561 in the microsomal membranes. The spectrophotometric data have been fully supported by Western analyses of the very same membrane fractions for expressed TCyT-b561 (Fig. 1). The H83-H156 pair of His residues coordinates the low-potential heme that is located on the cytoplasmic side of TCyT-b561 next to a predicted ASC-binding motive (Tsubaki et al. 2000) and assumed to be reduced by ASC.

When TCyT-b561 was labeled on its C-terminus by FLAG-tag (Flag) or His\_x-tag (C6H) or on its N-terminus by His\_y-tag (N6H), the protein expression level hardly decreased with the C6H tag but decreased by 50% and 75% with N6H and Flag tags, respectively (Fig. 1). The Western blot analyses (Fig. 1) supports the spectrophotometric data obtained with the His\_x-tagged proteins, but seems to overestimate the decrease in expression of the FLAG-tagged protein. It is very probably that the FLAG-tag at the C-terminus of TCyT-b561 interferes with the interaction between TCyT-b561 and its antibody.

Lysine mutants

Tsubaki et al. (2000) postulated an ASC-binding motive (Fig. 2) and a Lys residue (K85 in the bovine CGCyt-b561) as major components in ASC binding and ASC-dependent reduction of CGCyt-b561. Recent results with point-mutated recombinant mouse CGCyt-b561 (Bérczi et al. 2005), however, revealed the importance of the R72 residue (the R in the middle of the predicted ASC-binding motive) in the ASC-dependent reduction kinetics but did not verify the importance of the Lys residue, K83. It should be noted that K85 in the bovine CGCyt-b561 refers to the K83 in the mouse CGCyt-b561 and K80 in the TCyT-b561 sequence (Fig. 2). Also, R72 in the mouse CGCyt-b561 refers to R74 in the bovine CGCyt-b561. However, there is no R residue in the vicinity of the ASC-binding motive (Fig. 2, boxed area) in the Arabidopsis TCyT-b561 but there are K residues in TCyT-b561 at places where R residues are present in bovine or mouse CGCyt-b561 in the vicinity of the ASC-binding motive (K70 and K149 in TCyT-b561 as compared to R72 and R152 in mouse CGCyt-b561; see Fig. 2). Since the high-affinity binding of ASC to CGCyt-b561 has been connected to the presence of an R residue (namely of the R72 in mouse CGCyt-b561; Bérczi et al. 2005), we were interested in if any of the 5 K residues in the vicinity of these two R residues were able to play a distinguished role in ASC-dependent reduction kinetics of TCyT-b561.

When all 5 K residues (namely K70, K76, K79, K80, and K149; see Fig. 2) were one-by-one replaced by A, a minor but significant decrease (about 40%) in the specific content of TCyT-b561 in the yeast microsomal membrane fraction was observed only with the K80A mutant (Fig. 3). This observation was fully complemented by Western blot analyses (Fig. 3). As it was mentioned above, the K80 residue in Arabidopsis TCyT-b561 corresponds to K85 in the bovine and K83 in the mouse CGCyt-b561 sequences. Different experiments have attributed different importance to this particular lysine residue in ASC binding or in ASC-dependent reduction kinetics of CGCyt-b561 (Tsubaki et al. 2000; Bérczi et al. 2005). Furthermore, when the ASC-reducibility (in the presence of 25 mM ASC) to dithionate-reducibility (in the presence of 2.5 mM dithionite) – the so-called percentage ASC-reducibility – was compared, the lowest value was obtained with the K80A mutant (Fig. 3). These results obtained with the K mutants revealed that any K-to-A replacement in the vicinity of the ASC-binding motive in the CB domain could dramatically change neither the ASC-reducibility nor the expression of TCyT-b561.

In agreement with earlier kinetic studies on the recombinant mouse CGCyt-b561, the ASC-dependent reduction kinetics of all 5 K-to-A mutants could be explained only by assuming the presence of two “ASC-binding sites” with different affinity on the TCyT-b561:

$$[\text{Cyt} - b561]_{\text{reduc}} = \frac{A_1[AASC]}{K_1 + [AASC]} + \frac{A_2[AASC]}{K_2 + [AASC]},$$

where $A_1$ and $A_2$ are fitting parameters (referring to the reduction of either heme in TCyT-b561), $K_1$ and $K_2$ are the high-affinity and low-affinity binding constants, respectively. While $K_2$ seemed to be hardly influenced by any K-to-A replacement (data not shown), $K_1$ for the K80A mutant was significantly higher (about 10-fold) than that was for any other K-to-A mutant or for the non-mutated TCyT-b561 (Fig. 4). These results show that K80 in Arabidopsis TCyT-b561 plays a distinguished role in the ASC-dependent reduction kinetics of TCyT-b561.

In summary, H-to-A mutation of either His residues coordinating the high-potential heme in TCyT-b561 is lethal. H-to-A mutation at either His residues coordinating the low-potential heme in TCyT-b561 reduces significantly the expression level of the protein. Introduction of a His\_x-tag at the C-terminus of the TCyT-b561 decreases less the expression...
level than that of a His$_x$-tag at the N terminus or a FLAG-tag at the C terminus. The ASC-dependent reduction of the recombinant Arabidopsis TCyt-b$_{561}$ can be explained by assuming two “ASC-binding sites” with different affinity. It seems that K80 plays an important role in the ASC-dependent reduction of TCyt-b$_{561}$.

Acknowledgements

This work was supported by grants from NSF (IBN-0416742) and Hungarian Scientific Research Fund (OTKA T-034488). The authors are grateful for helps from Dan Su, Amy Vargas, and Daniel Griesen (Department of Biochemistry, UNL, Lincoln, NE, USA).

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