Volume 50(1-2):55-59, 2006
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Characterization of an ascorbate-reducible cytochrome *b*561 by site-directed mutagenesis

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ABSTRACT Ascorbate(ASC)-reducible cytochrome *b*561 (Cyt-*b*561) 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 Cyts-*b*561 identified by genomic analysis of *Arabidopsis thaliana* has been localized in the tonoplast. We have expressed the tonoplast-localized Cyt-*b*561 (TCyt-*b*561) in yeast (*Saccharomyces serevisiae*) cells and shown that the biophysical properties of the recombinant TCyt-*b*561 is very similar to those of the chromaffin granule Cyt-*b*561 (CGCyt-*b*561). 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_6-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-*b*561. These observations are discussed in comparison to properties of the recombinant CGCyt-*b*561.
Acta Biol Szeged 50(1-2):55-59 (2006)

KEY WORDS

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

Ascorbate(ASC)-reducible cytochromes *b*561 (Cyts-*b*561) are a newly described class of intrinsic trans-membrane proteins with two hemes (Wakefield et al. 1986a,b; Asard et al. 2001). Cyts-*b*561 have been identified in a great variety of organisms, including invertebrates, vertebrates, and plants as well (Verelst and Asard, 2003; Tsubaki et al. 2005). All Cyts-*b*561 are predicted to consist of six trans-membrane helices, the central four of them being identified as "Cyt-*b*561 (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-*b*561 proteins (Bashtovyy et al. 2003).

The only well characterized Cyt-*b*561 protein is the one in chromaffin granule membranes of neuroendocrine tissues, the CGCyt-*b*561. 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 monooxygenases such as dopamine β-hydroxylase and peptidyl glycine α-amidating monooxygenase (Kent and Fleming 1987; Seike et al. 2003). Both the natural and the recombinant form of the bovine CGCyt-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-*b*561 isoforms have been identified first in *Arabidopsis thaliana* (AtCyt-*b*561-1 through AtCyt-*b*561-4) 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-*b*561-1 (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-*b*561-1; Griesen et al. 2004), the recombinant protein has not yet been purified and charcterized. 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-*b*561-1 (hereafter called TCyt-*b*561) 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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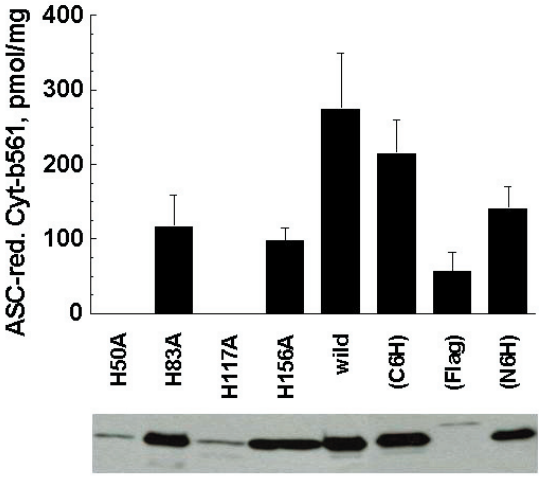

Figure 1. Expression analyses of H-to-A mutant and tagged recombinant TCyt-*b*561: the specific ASC-reduced TCyt-*b*561 content (top part) and by Western blot analyses (bottom part) of the yeast microsomal membranes. Experimental details are in the Materials and Methods. For Western blot analyses, 1 µg of membrane proteins was loaded in lanes for SDS-PAGE. (C6H), His_6-tagged TCyt-*b*561 at its C terminus; (N6H), His_6-tagged TCyt-*b*561 at its N terminus; (Flag), FLAG-tagged TCyt-*b*561 at its C terminus; wild, the recombinant Arabidopsis TCyt-*b*561 without any modification; HxA, H-to-A mutant of TCyt-*b*561 at the "x" position.

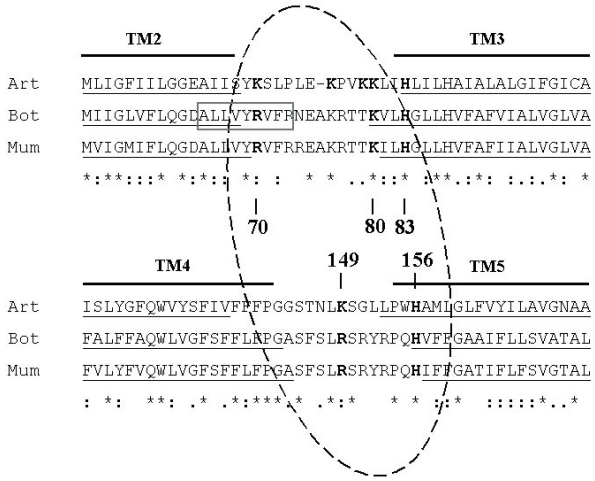

Figure 2. The vicinity (area inside the ellipses) of the ASC-binding motive (boxed area) in the CB domain (Pointing 2001). Partial sequences defining the CB domain are for Arabidopsis (*Arabidopsis thaliana*, Art) TCyt-*b*561, bovine (*Bos taurus*, Bot) CGCyt-*b*561, and mouse (*Mus musculus*, Mum) CGCyt-*b*561. Residue numbering refers to the sequence number in TCyt-*b*561. Bold face letters label residues discussed in the text. TM2 through TM5 label the trans-membrane helices of the CB domain. Underlined sequence regions label the predicted trans-membrane helix of the particular protein.

Materials and Methods

Plasmid construct and yeast transformation

Standard PCR methods were used to amplify the gene encoding AtCyt-*b*561-1 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 at 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 serevisiae*, 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) 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]-SPSPSPSVSNDDSVDFSYSAI) 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-*b*561 in the microsomal membrane vesicles were recorded in dual-wavelength mode (between 500 and 600 nm and reference at 601 nm; OLIS-

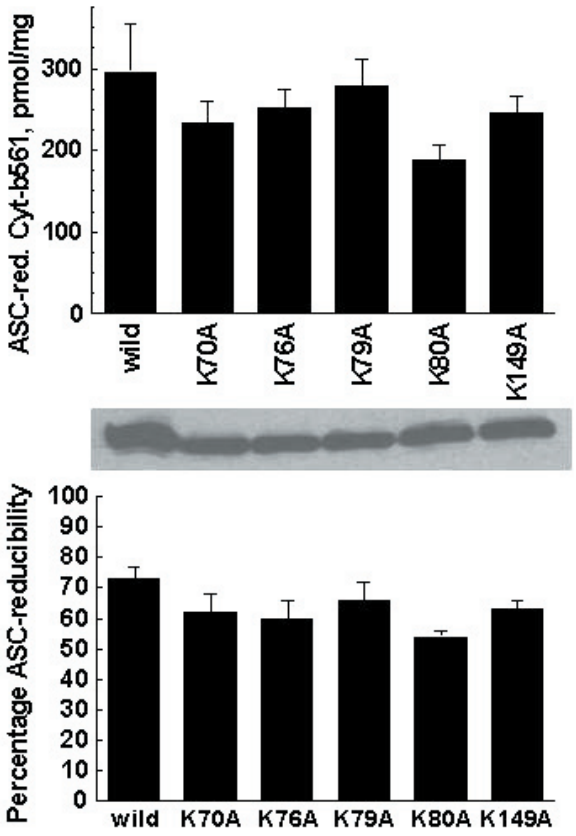

Figure 3. Specific content of K-to-A mutant TCyts-*b*561 in the yeast microsomal membrane fraction (top part), Western blot analyses of the mutants (middle part), and percentage ASC-reducibility of the mutants (bottom part). Experimental details are in the Materials and Methods. For Western blot analyses, 1 µg of membrane proteins was loaded in lanes for SDS-PAGE. KxA, K-to-A mutant of TCyt-*b*561 at the "x" position; wild, the recombinant Arabidopsis TCyt-*b*561 without any modification.

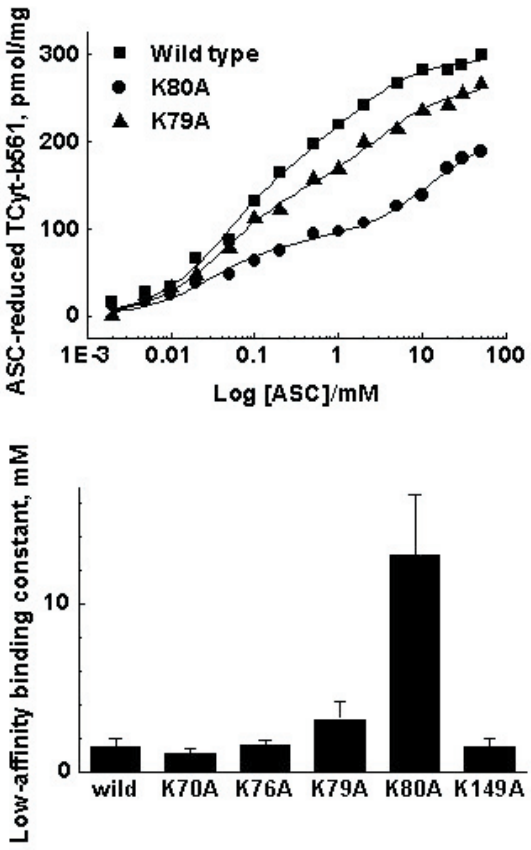

Figure 4. ASC-dependent reduction of TCyt-*b*561 (Wild type) and of K79A and K80A mutants of the wild type protein in the yeast microsomal membrane fraction (top part) and the "low-affinity" ASC-binding constant of TCyt-*b*561 (wild) and of all 5 K-to-A mutants of TCyt-*b*561 (bottom part). Experimental details are in the Materials and Methods. Membrane protein concentration in the optical cuvette was similar for all samples (2 ± 0.2 mg/ml). Top part: experimental points are from one series of experiments. Bottom part: averages with alteration from the average of two independent series of experiments.

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. Percentage ASC reduction is the ratio of ASC-reduced to dithionite reduced TCyt-*b*561 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-*b*561 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-*b*561 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 the two hemes in TCyt-*b*561 (Fig. 1). While the H-to-A replacement at positions of H50 and H117 resulted in total loss of detectability of TCyt-*b*561, the H-to-A mutation at positions of H83 and H156 resulted only in reduced specific content of TCyt-*b*561 in the microsomal membranes. The spectrophotometric data have been fully supported by Western analyses of the very same membrane fractions for expressed TCyt-*b*561 (Fig. 1). The H83-H156 pair of His residues coordinates the low-potential heme that is located on the cytoplasmic side of TCyt-*b*561 next to a predicted ASC-binding motive (Tsubaki et al. 2000) and assumed to be reduced by ASC.

When TCyt-*b*561 was labeled on its C-terminus by FLAG-tag (Flag) or His_6-tag (C6H) or on its N-terminus by His_6-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_6-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-*b*561 interferes with the interaction between TCyt-*b*561 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-*b*561) as major components in ASC binding and ASC-dependent reduction of CGCyt-*b*561. Recent results with point-mutated recombinant mouse CGCyt-*b*561 (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 appropriate Lys residue, K83. It should be noted that K85 in the bovine CGCyt-*b*561 refers to the K83 in the mouse CGCyt-*b*561 and K80 in the TCyt-*b*561 sequence (Fig. 2). Also, R72 in the mouse CGCyt-*b*561 refers to R74 in the bovine CGCyt-*b*561. However, there is no R residue in the vicinity of the ASC-binding motive (Fig. 2, boxed area) in the Arabidopsis TCyt-*b*561 but there are K residues in TCyt-*b*561 at places where R residues are present in bovine or mouse CGCyt-*b*561 in the vicinity of the ASC-binding motive (K70 and K149 in TCyt-*b*561 as compared to R72 and R152 in mouse CGCyt-*b*561; see Fig. 2). Since the high-affinity binding of ASC to CGCyt-*b*561 has been connected to the presence of an R residue (namely of the R72 in mouse CGCyt-*b*561; 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-*b*561.

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-*b*561 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-*b*561 corresponds to K85 in the bovine and K83 in the mouse CGCyt-*b*561 sequences. Different experiments have attributed different importance to this particular lysine residue in ASC binding or in ASC-dependent reduction kinetics of CGCyt-*b*561 (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-*b*561.

In agreement with earlier kinetic studies on the recombinant mouse CGCyt-*b*561, 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-*b*561:

$$[Cyt-b561]_{reduced} = \frac{A_1[ASC]}{K_1+[ASC]} + \frac{A_2[ASC]}{K_2+[ASC]},$$

where A_1 and A_2 are fitting parameters (referring to the reduction of either heme in TCyt-*b*561), K_1 and K_2 are the high-affinity and low-affinity binding constants, respectively. While K_1 seemed to be hardly influenced by any K-to-A replacement (data not shown), K_2 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-*b*561 (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-*b*561 is lethal. H-to-A mutation at either His residues coordinating the low-potential heme in TCyt-*b*561 reduces significantly the expression level of the protein. Introduction of a His_6-tag at the C terminus of the TCyt-*b*561 decreases less the expression

level than that of a His_6-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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