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Ascorbate-reducible *b*-type cytochrome in the plant plasma membrane

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ABSTRACT It has been know for 20 years that the plasma membrane (PM) in plants contains more than one kind of *b*-type cytochromes. One of them has rather high redox potential (can fully be reduced by ascorbate) and is capable of electron transport through the PM. Three *b*-type cytochromes have recently been predicted from the full genome of *Arabidopsis thaliana.* In order to identify and characterize the one located in the PM, first PM vesicles were purified from *Arabidopsis* leaves, then the PM vesicles were solubilized and the fully ascorbate-reducible *b*-type cytochrome was partially purified. Redox titration of the partially purified *b*-type cytochrome revealed the presence of two hemes with redox potentials higher than 100 mV. The major polypeptide band of this fraction on SDS-PAGE was at ~120 kDa. This value is much higher than the apparent molecular mass of either the fully ascorbate-reducible *b*-type cytochrome purified from *Phaseolus* hypocotyls or the cyt. *b*-561 proteins purified from chromaffin granule membranes or the calculated molecular masses for the three polypeptides predicted from the full genome of *Arabidopsis.*

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The idea that not only ATP-hydrolyzing proteins (ATPases) but also reduced electron transport chains might contribute to the energization of the plasma membrane (PM) was first formulated and discussed about 20 years ago (Crane et al. 1985). Since that time numerous redox proteins (mostly NAD(P)H-oxidizing proteins) have been identified and partially purified from plant PM, however, neither of them seemed to be capable of *trans*-PM electron transport (Lüthje et al. 1997; Bérczi and Møller 2000). At the beginning of 1990s it was shown that a *b*-type cytochrome (cyt *b*) is located in the plant PM which itself is capable of *trans*-PM electron transport (Asard et al. 1992). Three years ago it was shown that the *FRO2* gene encodes for a putative *trans*-PM flavo-cytochrome capable of *trans*-membrane electron transport (Robinson et al. 1999). The first hypothesis of the presence of a reduced electron transport chain in the plant PM (in which a cyt *b* and an NAD(P)H dehydrogenase might participate) was outlined after vitamin K had been identified in the PM (Döring and Lüthje 1996).

Optical redox titration of cyts *b* in the plant PM vesicles has revealed the presence of more than one kind of cyt *b* (Asard et al. 1998). The major component can be fully reduced by ascorbate and it is the component responsible for the *trans*-membrane electron transport. An ascorbate-reducible (asc-red.)and electron-transporting cyt *b* (cyt b_{561}) has long been known to be present in the chromaffin granule membrane of mammalian adrenal cells (Flatmark and Terland 1971; Apps et al. 1980). The presence in plants of a protein homologous to cyt b_{561} has been suggested after identifying of three genes in the genome of *Arabidopsis thaliana* (L.) Heynh encoding for polypeptides which show high structural similarity to the mammalian cyt b_{561} (Asard et al. 2001). Attempts for the purification of the fully asc-red. cyt *b* from plant PM (called also plant PM cyt b_{561}) have been successful only recently (Scagliarini et al. 1998). However, the apparent molecular mass as well as the anion-binding characteristics of the plant polypeptide differed considerable from those of the mammalian protein (Trost et al. 2000). Moreover, the protocol developed for the purification of cyt b_{561} from the bean hypocotyl PM did not work in case of *Arabidopsis* leaf and maize root PM vesicles (Bérczi et al. 2001).

In this paper we present a protocol for purifying a fully ascorbate-reducible cyt from *Arabidopsis* leaf PM which has two hemes with redox potential higher than 100 mV each. Relation of this cyt *b* to the other ones purified and characterized earlier from different objects will be also discussed.

Materials and Methods

Arabidopsis thaliana (L.) Heynh (ecotype Columbia) was grown under controlled conditions (Bérczi et al. 2001). Leaves of 9-week-old seedlings (seedlings just before flowering) were harvested and immediately used for PM preparation. PM vesicles were prepared by phase partitioning (Larsson et al. 1994) and then solubilized by non-ionic detergents. Solubilized PM proteins were separated by two consecutive ion exchange chromatography steps; firstly on a Mono-Q column (HR 5/5, Pharmacia, Sweden; strong anion exchanger) and secondly on a Mono-P column (H/R 5/20, Pharmacia, Sweden; weak anion exchanger). Between the two chromatography steps, the fractions containing the asc-red. cyt *b* were concentrated by centrifugation using Centrex UF concentrators (Schleicher and Schuell, Germany) with 10 kDa cut-off filters and the buffer of concentrated and combined fractions were exchanged using a PD-10 column (Amersham Pharmacia Biotech, England). Final fractions were also concentrated and washed with the storage buffer [25 mM bis-Tris, pH 7.0, 0.5 mM betaine, 1 % (w/v) glycerol, 0.05% (w/v) $C_{12}E_9$] twice by using Centrex UF concentrators (see above).

The activity of both the positive (K^+-stimulated and

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vanadate-inhibited Mg^{2+}-ATPase, glucan-synthase II [GS-II]) and the negative (cytochrome *c* oxidase [CCO], Mg^{2+}-IDPase) marker enzymes of the PM fraction was determined as given earlier (Bérczi et al. 1989 and references therein).

Optical redox titration was carried out with the partially purified proteins (the Mono-Q fractions containing the fully asc-red. *b*-type cytochromes) in 50 mM MOPS, pH 7.0, 0.5 % (w/v) glycerol, 0.025 % (w/v) $C_{12}E_9$, under anaerobic conditions (*e.g.* under argon atmosphere) and continuous stirring. Redox mediators were as in Trost et al. (2000) but in 4 times higher concentrations.

Concentration of cyt *b* was determined spectrophotometrically (Asard et al. 1989). Spectra were recorded at room temperature in the absence and presence of ascorbate or ascorbate plus dithionite against either an appropriate buffer solution or the same amount of ferricyanide-oxidized cyt *b*. For calculations, the absorbance at the a-band maxima of difference spectra and a molar extinction coefficient of 20 $mM^{-1}cm^{-1}$ were used.

Estimation of the molecular mass of proteins was performed by SDS-PAGE in the buffer system of Laemmli (1970) and a Mini-Protean apparatus (Bio-Rad, USA). Polypeptide bands were visualized by silver staining using broad molecular weight markers and the Silver Stain Plus Kit (both from BioRad, USA).

Results and Discussion

First the purity of the PM vesicles' fraction was determined. The positive marker enzymes (K^+-stimulated and vanadate-inhibited Mg^{2+}-ATPase, GS-II) were enriched while the mitochondrial marker enzyme (CCO) was almost completely depleted in the PM fraction as compared to that in the microsomal fraction. Hardly any Mg^{2+}-activated IDPase and nitrate-inhibited Mg^{2+}-ATPase activity could be found in both the MF and the PM fraction. It was concluded that the purity of the PM fraction corresponded to the widely-accepted international standards.

In contrast to the bean protein, the highest yield of the solubilized asc-red. cyt *b* was obtained with mixture of $C_{12}E_9$ non-ionic detergent and 1-Octanol (20:1 w:w) amongst the detergents and solubilization conditions tested.

In contrast to the bean protein, the asc-red. cyt *b* from *Arabidopsis* leaves bound to Mono-Q at every pH tested between 5 and 9. However, independently of the pH, asc-red. cyt *b* always eluted between 50 and 100 mM KCl. SDS-PAGE analysis revealed that asc-red. cyt *b*-containing Mono-Q fractions contained many polypeptides, so that a further purification step was required for identifying the apparent molecular mass of the protein in question.

Mono-P bound the asc-red. cyt. *b* which did not elute with the pH gradient but eluted with a salt was step applied after the pH gradient. Asc-red. cyt *b*-containing Mono-P fractions contained only one polypeptide with apparent molecular mass of ~120 kDa.

Redox titration of the partially purified fraction containing the asc-red. cyt. *b* revealed the presence of two hemes with redox potentials of 135 mV and 180 mV. These values are in good agreement with results obtained for other asc-red. cyts *b* (Trost et al. 2000).

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