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Rapid report

Purification of cytochrome *b*-561 from bean hypocotyls plasma membrane. Evidence for the presence of two heme centers

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Abstract

The high potential, ascorbate-reducible *b*-type cytochrome of plant plasma membranes, named cytochrome *b*-561, has been purified to homogeneity from etiolated bean hypocotyls. The pure protein migrated in denaturing electrophoresis as a broad band of approximately 55 kDa, and was found to be glycosylated. Optical redox titrations of partially purified cytochrome *b*-561 indicated that it contains two hemes with similar spectral features, but distinct midpoint redox potentials (E_{m7}+135 mV and +206 mV, respectively). The presence of two heme centers in cytochrome *b*-561 is consistent with its role in electron transfer across plant plasma membranes.

Keywords: Cytochrome *b*-561; Ascorbate; Plasma membrane redox; Redox titration; *Phaseolus vulgaris*

The presence of *b*-type cytochromes in the plasma membrane (PM) of higher plants has long been demonstrated in several tissues and species (for a review, see [1]). Early studies pointed to a cytochrome species liable to reduction by blue light, a redox event known as light induced absorbance change [2,3]. It was later realized that the cytochrome reduced by blue light was indeed a major, high potential *b*-type cytochrome of the PM, and that it was apparently ubiquitous in plants [4].

Optical redox titrations of purified PM preparations have set the basis for distinction between different *b*-type cytochromes associated with this membrane. In spite of some discrepancies among species, it was apparent that plant PM generally contains one or two high potential, *b*-type cytochromes (E_{m7} +70 to +220 mV), plus an additional low potential component (E_{m7} −23 to −80 mV), tentatively labelled as cytochrome *b*-5 [4–6]. Recently, a method of purification of a high potential *b*-type cytochrome from bean hypocotyls PM has been devised [7]. The purified cytochrome was fully reduced by ascorbate and it was named cytochrome *b*-561 (cyt *b*-561) because of the α-band maximum at 561 nm. No other cytochromes of plant PM have been isolated thus far.

Since ascorbate can reduce a major *b*-type cytochrome from either side of the PM, it is believed that cyt *b*-561 is a transmembrane protein. Accordingly, by use of ascorbate-loaded PM vesicles in

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right-side-out orientation, cyt *b*-561 was shown to be involved in the trans-PM electron transport from internal ascorbate to an external electron acceptor, such as monodehydroascorbate (MDA) or ferricyanide [8]. cyt *b*-561 is the only redox protein of the plant PM whose involvement in a transmembrane electron transfer has been directly demonstrated. A comparison of the ascorbate-mediated electron transport properties suggested that plant cyt *b*-561 may be homologous to animal cyt *b*-561, which is involved in the regeneration of ascorbate in chromaffin granules of adrenal medulla and other cells [1,9,10]. This latter cytochrome is a transmembrane protein binding two hemes [11–16].

In the present work, the published purification procedure for cyt *b*-561 of etiolated bean hypocotyls [7] has been applied with few modifications (for details, see legend to Fig. 1). In brief, purified PM vesicles were diluted in a hypo-osmotic buffer to release contaminating soluble proteins, and cyt *b*-561 was solubilized from the resulting membrane fraction by treatment with Triton X-100 (reduced form). Solubilized cyt *b*-561 was then purified by two consecutive anion exchange steps at pH 8 and pH 10, respectively. Purified cyt *b*-561 showed a typical reduced minus oxidized spectrum with peaks at 429, 530 and 561 nm [7].

Purified cyt *b*-561 migrated in SDS-PAGE as a single, silver-stained band corresponding to an apparent molecular mass of approximately 55 kDa (Fig. 1, lane A). The band was broad and diffuse. The silver-stained 55-kDa band corresponded to a weak signal in gels treated for heme detection [17]. However the strong band co-migrating with the front-line indicated that most of the heme was detached from the apoprotein under denaturing conditions, as expected for the non-covalent type of interaction typical of *b*-type cytochromes (Fig. 1, lane C). The diffuse aspect of the cyt *b*-561 band in SDS-PAGE prompted us to check if the protein was glycosylated. Following sugar specific staining (Glycoprotein Detection kit, Sigma, USA) the appearance of a band around 55 kDa strongly suggested that cyt *b*-561 was indeed glycosylated (Fig. 1, lane B). It should be noted that glycosylation may lead to a significant overestimation of the cyt *b*-561 apparent molecular mass. Moreover, the combination of hydrophobicity (detergent requirement for solubiliza-

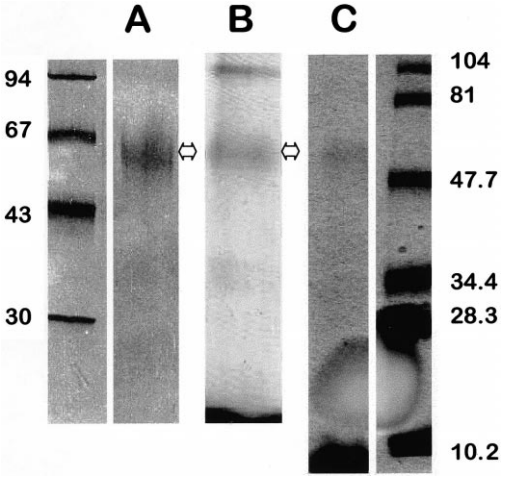

Fig. 1. SDS-PAGE analysis of purified cyt *b*-561 from bean hypocotyls PM. cyt *b*-561 was purified by a simplified version of the procedure of Scagliarini et al. [7]. PM vesicles were obtained from etiolated bean hypocotyls by two-phase partitioning [4,25]. Before solubilization, PM vesicles were diluted 10-fold in 20 mM Tris–HCl, pH 8.0, recovered by centrifugation (167 000×*g*, 30 min) and resuspended in solubilization buffer (20 mM Tris–HCl, pH 8.0, 1% (v/v) glycerol, 330 mM sucrose). Washed PM's were solubilized by Triton X-100 (reduced form, 2 mg detergent per mg protein) under stirring for 1 h at room temperature. Following centrifugation to remove unsolubilized material (82 000×*g*, 30 min), the supernatant was loaded on a Q-Sepharose anion exchange column (Pharmacia, Sweden) equilibrated with 20 mM Tris–HCl, pH 8.0, 1% (v/v) glycerol, 0.1% (w/w) reduced Triton X-100. Under these conditions, cyt *b*-561 (as detected by ascorbate reduced minus ferricyanide oxidized differential spectra) eluted with the column flow-through, and was immediately desalted by a Hi Trap Fast Desalting cartridge (Parmacia, Sweden) equilibrated with 20 mM 3-[cyclohexaamino]-1-propanesulfonic acid (CAPS), pH 10.0, 1% (v/v) glycerol, 0.1% (w/w) reduced Triton X-100. Finally, the sample was loaded on an anion exchange Resource Q column (Pharmacia, Sweden) equilibrated with the latter buffer, and eluted with a linear KCl gradient (0–0.5 M). The central portion of the cyt *b*-561 elution peak was electrophoretically analyzed. SDS-PAGE was performed according to [26]. Lane A: purified cyt *b*-561 was silver-stained by a procedure including silver diamine [27]. Marker proteins (kDa) run on the same gel are shown on the left. Lane B: a similar sample as in lane A was stained by the Sigma Glycoprotein Detection kit based on the reaction with periodate. A glycosylated polypeptide of about 100 kDa was also detected in this cyt *b*-561 preparation. Lane C: the gel was loaded with purified cyt *b*-561 and stained for heme-associated peroxidase activity according to [17]. Note that this method also detected the heme released from the protein during the sample preparation procedure, as shown by the marked spot at the end of the run. Marker proteins (kDa) run on the same gel are shown on the right.

tion), glycosylation, and the alkaline isoelectric point (based on the chromatographic behavior [7]) may be the reason for cyt *b*-561 being relatively insensitive to staining by either silver nitrate or Coomassie [7].

Spectrophotometric redox titrations were carried out with partially purified preparations of cyt *b*-561, following the anion exchange chromatography at pH 8.0 of solubilized PM proteins. No other cytochrome species were detected following complete purification of cyt *b*-561 from this material [7]. On the other hand, the low final yield of homogeneous cyt *b*-561 prevented collecting sufficient amounts of pure protein for optical redox titrations.

The differential absorption spectra of cyt *b*-561 were recorded under under anaerobic conditions at variable redox potentials [18], as shown in Fig. 2. Both the shape and the wavelength maxima of the α and β bands (530 and 561 nm, respectively) did not change significantly as a function of the ambient redox potential. Experimental data were interpolated by nonlinear regression analysis using alternative nernstian equations based on the existence of either one or two heme components. In each experiment the latter condition led to a better fit of the data (i.e. higher coefficients of variation) strongly suggesting the presence of two hemes with different midpoint redox potentials. These were 135 ± 29 mV and 204 ± 34 mV, respectively (mean ± S.D. of three independent redox titrations). The relative abundance of the two hemes was calculated to be similar, with the lower potential heme being on average slightly less abundant (40%) than the high potential one (60%).

The midpoint redox potentials of purified cyt *b*-561 from bean hypocotyls PM are in good agreement with previous determinations made on intact PM vesicles. Using the same plant material as in this work, Asard et al. [4] resolved three *b*-type hemes in purified PM vesicles, namely two major forms at +119 and +226 mV, and a minor component at −43 mV. As previously demonstrated [7,19], the low potential component, possibly a cytochrome *b*-5 isoform, was efficiently separated from the high potential hemes by means of an anion exchange chromatographic step at pH 8.0. It remained however an open question whether the two remaining high potential hemes belonged to a single or two distinct cytochrome species. In this work we show

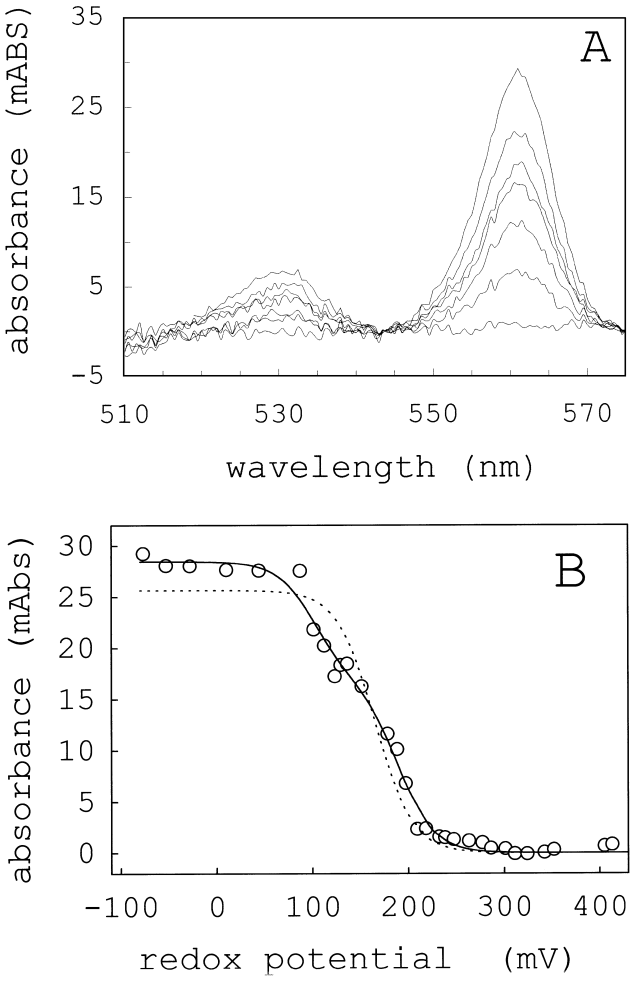

Fig. 2. Potentiometric redox titration of partially purified cyt *b*-561. cyt *b*-561 was partially purified from etiolated bean hypocotyls by anion exchange chromatography (pH 8.0) of solubilized PM proteins. Absorption spectra were recorded with a Jasco 7850 spectrophotometer. Redox titrations were performed by the method of Dutton [18] with ferricyanide as an oxidant and either ascorbate or dithionite as reductants, in the presence of the following mediators (E_{m7}): 5 μM *p*-benzoquinone (+280 mV), 5 μM diaminodurole (+240 mV), 2.5 μM 1,2-naphthoquinone (+145 mV), 2.5 μM phenazine methosulfate (+80 mV), 5 μM 1,4-naphthoquinone (60 mV), 2.5 μM phenazine ethosulfate (+55 mV), 5 μM duroquinone (+5 mV). (A) Representative reduced minus oxidized absorbance spectra in the 510–575 nm region. From top to bottom, the spectra were recorded at redox potentials of −77, +101, +118, +153, +188, +197, +413 mV, respectively. (B) The absorbance changes at 561–575 nm (A) were plotted against the redox potential. Theoretical curves are generated by non linear regression (Sigma Plot) using the Nernst equation for either one (dashed line, $r = 0.9671$) or two heme components (solid line, $r = 0.9921$).

that the two major, high potential heme centers detected in intact PM vesicles belong indeed to one and the same cytochrome species, named cyt *b*-561.

The evidence that cyt *b*-561 allocates two heme centers is consistent with the observation that cyt *b*-561 in PM vesicles may drive an electron transfer across the PM, since two redox centers at least seem necessary to span the hydrophobic core of phospholipid bilayers. The midpoint redox potentials of the two heme centers are also consistent with ascorbate (E_{m7} +60 mV [20]) being the reductant for the lower potential heme (E_{m7} +135 mV), while the higher potential heme (E_{m7} +204 mV) may act as reductant for MDA (E_{m7} +330 mV [20]). The nature of the electron donors and acceptors of plant cyt *b*-561, has suggested a possible relationship with cyt *b*-561 of animal chromaffin granules [1]. The latter utilizes cytosolic ascorbate to regenerate ascorbate from MDA inside the chromaffin granule, thereby sustaining the ascorbate-dependent biosynthesis of dopamine and noradrenaline [9,10]. Animal cyt *b*-561 binds two hemes by means of two histidine pairs [12–15]. Both hemes are characterized by positive redox potentials (E_{m7} = +70 mV and +170 mV, respectively [21]). Purified animal cyt *b*-561 was shown to catalyze on its own an electron transfer across artificial lipid bilayers [11], and molecular modelling suggests that the two hemes face opposite sides of the membrane, then being accessible to the hydrophilic molecules ascorbate and MDA [16]. Recently, two *Arabidopsis* genes were found to be homologous to animal cyt *b*-561 (Atb561-A, [22]; Atb561-B, [23]) and *in silico* analysis of these sequences predicted a similar transmembrane topology as well as the coordination of two distinct hemes. Moreover, the protein product of Atb561-A was predicted to be glycosylated [1]. It is therefore of interest that cyt *b*-561 from bean hypocotyls PM is glycosylated, it allocates two hemes and shares with animal cyt *b*-561 the capability to use ascorbate and MDA as electron donor and acceptor, respectively [24]. On the other hand, the putative cyt's *b*-561 from *Arabidopsis* have calculated molecular masses of ~26 kDa, and animal cyt *b*-561 is ~28 kDa, while the apparent molecular mass of purified cyt *b*-561 from bean hypocotyls was around 55 kDa. This is a rather large discrepancy even for a glycoprotein. Unfortunately we did not succeed in enzymatically removing the oligosaccharides from the protein. However, human purified cyt *b*-561 at least in one case [21] showed a marked tendency to aggregate under denaturing conditions, and to migrate in SDS-PAGE as an additional band of 43 kDa. For the time being we conclude that cyt *b*-561, the prominent cyt species of plant PM, is a glycosylated bis-heme transmembrane cytochrome with similar features as animal cyt *b*-561 of animal chromaffin granules. By analogy, the role of this redox protein in plants may be related to the control of the ascorbate redox state in the cell wall.

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