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Transmembrane electron transport in ascorbate-loaded plasma membrane vesicles from higher plants involves a *b*-type cytochrome

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The possible involvement of a high-potential *b*-type cytochrome in plasma membrane electron transport was tested using ascorbate-loaded membrane vesicles. Absorption spectra demonstrated that the cytochrome was about 89% reduced in these preparations. Use of ascorbate oxidase and washing of the vesicles further indicated that reduction was mediated by intra-vesicular ascorbate. Addition of low concentrations of ferricyanide caused a rapid cytochrome oxidation followed by a slower re-reduction. The kinetics of this response indicate that the electron acceptor was fully reduced before re-reduction of the cytochrome occurred. These observations suggest that the *b*-type cytochrome mediates transmembrane electron transfer.

Plasma membrane; Electron transport; Ascorbic acid; Cytochrome *b*; *Phaseolus vulgaris* L.

1. INTRODUCTION

Electron transport reactions at the plasma membrane of plant cells have been implicated in a variety of physiological responses, including ion uptake, hormonal growth control and pathogen defense [1]. At least two distinct NAD(P)H-dependent oxido-reductases have been demonstrated in isolated plasma membranes and are in the process of being purified [2,3]. In addition to these enzymes the plasma membrane also contains a particular *b*-type cytochrome. Redox titration experiments in membrane fractions from six higher plant species [4–6] resulted in the characterization of a specific *b*-type cytochrome with a midpoint redox potential (E'_0) between +120 and +160 mV as the major component. The α-band was located at 560–561 nm [5,6] and sodium ascorbate acts as an *in vitro* electron donor to this cytochrome. The presence of still other, minor cytochrome components in the plasma membrane was also suggested, possibly including cytochrome b_5 (E'_0 from 0 to −57 mV) [5,6]. Although a possible participation of the high-potential *b*-type cytochrome in blue light perception has been suggested [7], no definite physiological function could be assigned to either of these cytochromes.

Recently a method became available for the preparation of ascorbate-loaded plasma membrane vesicles, capable of electrogenic transmembrane electron transport [8,9]. These membrane fractions provide a tool to investigate the possible involvement of the high potential *b*-type cytochrome in trans-plasma membrane electron transport in plants. This paper provides the first experimental support for this hypothesis and indicates striking similarities to the suggested operation of cytochrome b_{561} of adrenal chromaffin granules (E'_0 +140 mV) [10,11].

Abbreviations: E'_0, redox potential at pH 7.0; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PEG, poly(ethylene glycol).

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2. MATERIALS AND METHODS

Hypocotylar hooks of 5-day-old etiolated bean (*Phaseolus vulgaris* var. 'Limburgse Vroege') were harvested and collected on ice. Generally 100 g of tissue was homogenized in 250 ml of cold HEPES-KOH buffer (330 mM sucrose, 50 mM HEPES, 0.1% BSA at pH 7.5) supplemented with 1 mM DTT, 0.5 mM PMSF and 0.3% insoluble PVP. Plasma membranes were purified by partitioning in an aqueous two-phase system as described earlier [5], except for the use of 330 mM sucrose in all buffers. Plasma membrane pellets were resuspended in 330 mM sucrose, 25 mM HEPES-KOH at pH 7.5 to a final concentration of about 5 mg protein·ml^{-1} and kept on ice. To prepare vesicles loaded with ascorbate, 100 mM sodium ascorbic acid was added to the homogenisation buffer immediately before use, and the pH readjusted with KOH. Measurements using ascorbate-loaded vesicles were performed within 4–6 h of preparation.

Spectrophotometric determinations were carried out on an Aminco DW2a dual-wavelength spectrophotometer in 600 μl samples. Cytochrome *b* absorption spectra were scanned at 2 nm·s^{-1} (Fig. 2) or 10 nm·s^{-1} (Fig. 3) at room temperature, relative to 570 nm (isosbestic point). Reduction of ferricyanide was recorded at 420 nm (isosbestic point for the cytochrome) relative to 500 nm. All measurements were performed in resuspension buffer with additions as indicated in the figure legends.

Protein concentration was estimated by the method of Markwell [12] in BSA-free membrane preparations and extrapolated to concentration used in the assays.

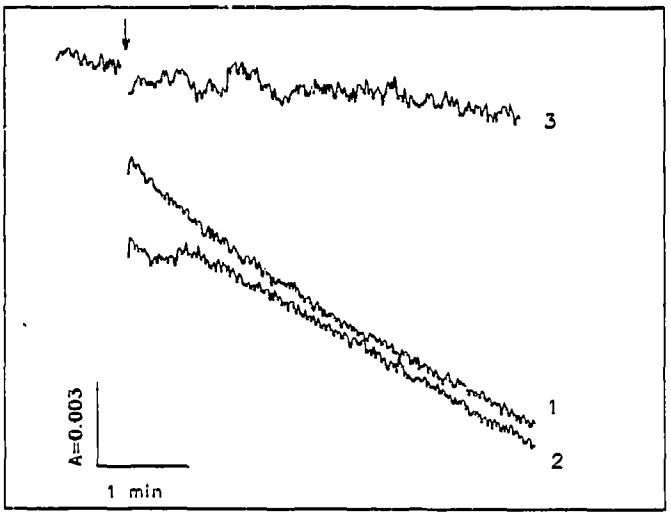

Fig. 1. Reduction of ferricyanide (0.5 mM, recorded at 420 nm) by the addition of ascorbate-loaded vesicles (arrow, 0.25 mg protein) in the presence (1) and absence (2) of ascorbate oxidase (1.6 U), and by ascorbate-free vesicles (3).

3. RESULTS

3.1. *Membrane preparation and electron transport*

Partitioning of a crude membrane preparation from bean hypocotyls in an aqueous two-phase system results in a highly enriched plasma membrane fraction with mainly right-side-out oriented vesicles [5]. The purity of this preparation was recently confirmed by electron microscopy after specific phosphotungstic acid staining and calculated to be about 99% (D. Lambrechts, personal communication).

The tissue homogenisation buffer was supplemented with 100 mM sodium ascorbate to include high concentrations of the reductant inside the plasma membrane vesicles [8,9]. The membrane-impermeable electron acceptors, ferricyanide and cytochrome *c*,were rapidly reduced by the ascorbate-loaded membranes (Fig. 1, not shown for cytochrome *c*). Addition of ascorbate oxidase did not affect the reduction rates nor did ascorbate-free vesicles support the reduction of either electron acceptor (Fig. 1).

3.2. *Cytochrome* b *reduction in ascorbate-loaded plasma membrane vesicles*

The absorption spectrum of the ascorbate-containing plasma membrane vesicles clearly shows the α-band maximum of the reduced *b*-type cytochrome near 561 nm (Fig. 2). Addition of sodium ascorbate or sodium dithionite to the vesicle suspension resulted in a further increase of the absorption band. These results are consistent with previous findings [5] that indicate the presence of a maximum of 50–60% (relative to the dithionite-reducible cytochromes) of a +120 mV ascorbate-sensitive cytochrome in bean hypocotyl plasma membranes. Approximately 89% of this component was found reduced in the freshly prepared ascorbate-loaded vesicles.

Addition of ascorbate oxidase (16.5 U) resulted in a slight irreversible reoxidation of the cytochrome (Fig. 2). Washing of the ascorbate-loaded membranes by repelleting and resuspension under isoosmotic conditions did not affect the level of reduction of the cytochrome, whereas vesicle disruption by osmotic shock in the presence of ascorbate oxidase (1.6 U) caused a rapid and complete reoxidation of the cytochrome (not shown). Since both ascorbate oxidase and the fully reduced ascorbic acid should be considered impermeable, and since ascorbate leakage is insignificant up to 6 h after vesicle preparation [9], these observations indicate that the cytochrome was mainly reduced by ascorbate effectively trapped inside the vesicles.

3.3. *Cytochrome oxidation by extra-vesicular electron acceptors*

Experiments with ascorbate-loaded chromaffin vesicles have demonstrated that reduction of externally added electron acceptors by intra-vesicular ascorbate is mediated by a high potential *b*-type cytochrome [10]. A

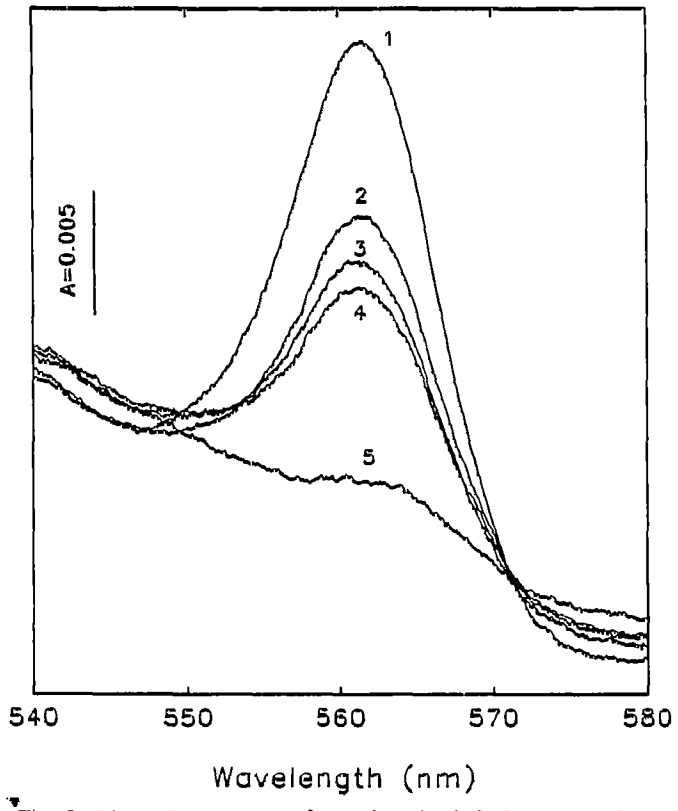

Fig. 2. Absorption spectra of ascorbate-loaded plasma membrane vesicles (1.5 mg protein), with the following additions: (1) sodium dithionite (solid); (2) sodium ascorbate (310 nmol at pH 7.5); (3) no addition; (4) ascorbate oxidase (16.5 U); and (5) ascorbate oxidase and ferricyanide (100 nmol).

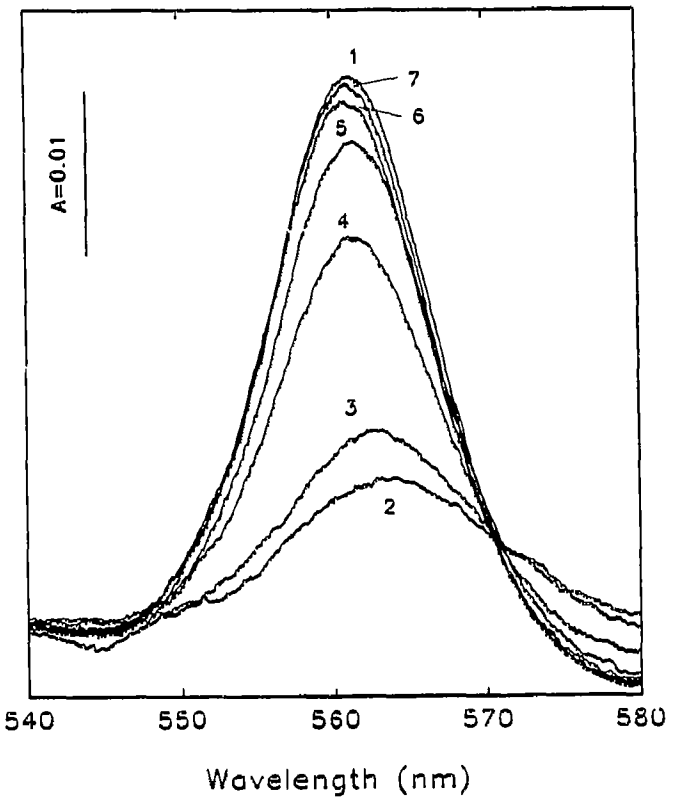

Fig. 3. Changes in the α-band absorption maximum of the *b*-type cytochrome recorded before (1) and immediately after (2) addition of 10 nmol ferricyanide. Subsequent recordings were at approximately 20 s time intervals (3–7). Protein concentration 3 mg; ascorbate oxidase 3.2 U.

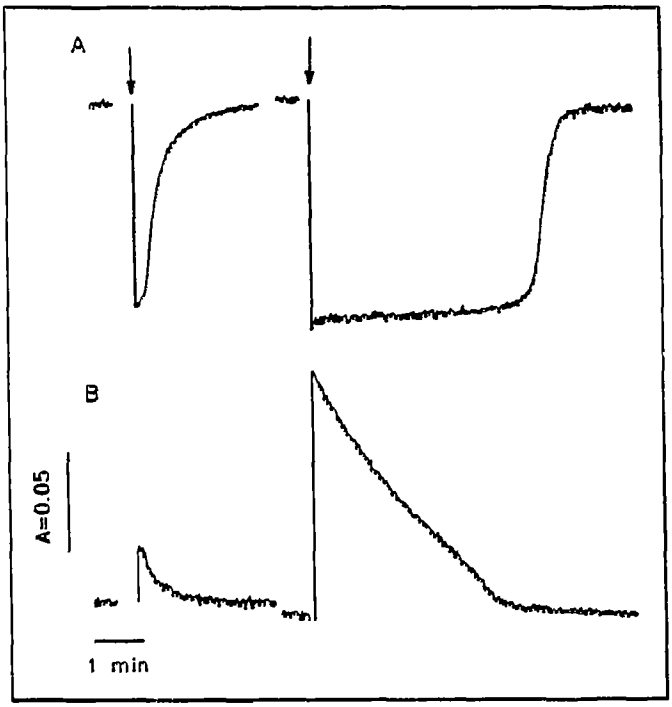

Fig. 4. Time–course of the α-band absorption change (A) and ferricyanide reduction (B) (recorded at 561 nm and 420 nm, respectively). Ferricyanide additions were 1 nmol (first arrow) and 10 nmol (second arrow) in the presence of ascorbate oxidase (3.2 U). Protein concentration 1.5 mg.

cytochrome with comparable redox potential as higher plant plasma membranes was therefore a likely candidate to support a similar function in the above mentioned transmembrane electron transport reactions.

Addition of low amounts of ferricyanide to the ascorbate-loaded vesicles caused a rapid oxidation of the *b*-type cytochrome followed by a slower re-reduction (Fig. 3). Inclusion of 3.2 U of ascorbate oxidase ensures that cytochrome reduction is mediated by intra-vesicular ascorbate only. Ferrocyanide was completely unable to induce this response. The time–course of the reaction was monitored at 561 nm relative to 570 nm (Fig 4A). Increasing ferricyanide concentrations resulted in an increased lag-phase with virtually no effect on the rate of the subsequent cytochrome reduction. The duration of the initial lag-phase also corresponded closely to the time needed to fully reduce the same amount of ferricyanide (Fig. 4B), indicating that ferricyanide reduction actually precedes the cytochrome re-reduction. High amounts of ferricyanide (>500 nmol) resulted in the irreversible reoxidation of the cytochrome (see Fig. 2). The nature of the residual cytochrome *b* absorption with a maximum near 563 nm is still unclear (Fig. 2 and Fig. 1).

4. DISCUSSION

Purified plasma membrane vesicles loaded with sodium ascorbate provide a means to investigate transmembrane electron carriers. Of particular interest in this respect is the high-potential *b*-type cytochrome present in all higher plant cells so far tested. The presented data indicate that ascorbate reduces about 80% of this cytochrome from the interior (=cytoplasmic side) of the vesicles. These vesicles are also capable of transferring electrons from internal ascorbate to an externally added impermeable electron acceptor, such as ferricyanide and cytochrome *c*.

The rapid oxidation of the cytochrome upon addition of ferricyanide and in the presence of ascorbate oxidase, indicates the transfer of electrons on the extra-vesicular membrane face. Since the cytochrome is only reduced from the vesicle interior, this process strongly suggests a transmembrane electron transfer with the cytochrome operating as an electron carrier. Also the redox potential of the major plasma membrane cytochrome (E'_0 around +120 mV) makes it a suitable intermediate in the electron transfer from ascorbate (+80 mV) to ferricyanide (+430 mV).

The results from this work all strongly point to similarities to the action of a high-potential *b*-type cytochrome in chromaffin granule membranes and neurose-

cretory vesicles (E'_0 +140 mV, α-band at 561 nm). In vitro electron transport using ascorbate-loaded granules shows qualitatively identical changes in the redox state of the cytochrome [10]. Quantitative differences are probably related to the 10-fold lower cytochrome concentration in the plant plasma membrane and to differences in vesicle volumes. Chromaffin granule cytochrome b_{561} mediates the regeneration of intra-vesicular ascorbate free radicals from cytosolic ascorbate [13].

Ascorbic acid in the cytosol of plant cells may well act as a natural electron donor to the high-potential cytochrome. The nature of the electron acceptor on the cell wall side of the membrane is, however, unclear. Since extracellular ascorbate oxidase has been demonstrated in plant tissues the transmembrane electron transport could possibly be related to ascorbate metabolism in the cell wall matrix [14]. Alternatively, with molecular oxygen as an electron acceptor, superoxide radical and hydrogen peroxide may be formed which could possibly be involved in pathogen defense reactions and cell wall biosynthesis [1].

Transmembrane electron transport could also be mediated by NADH generated inside isolated plasma membrane vesicles [9]. However, since the reduced nucleotide is a poor electron donor to the high-potential *b*-type cytochrome, the electron transfer pathway is possibly different from the one identified in our work.

The assay system described in this paper will further support the functional characterization of the high-potential, plasma membrane *b*-type cytochrome.

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