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Ion transport across chloroplast inner envelope vesicles

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Introduction

The ionic environment of chloroplasts is important for photosynthesis, nitrogen metabolism and sulfur metabolism, yet how this internal environment is regulated is still not known. Isolated chloroplast inner envelope membranes are competent for transport studies. These membranes can be manipulated to form vesicles of largely right side-out and inside-out orientation and both the intravesicular and extravesicular buffer contents can be controlled. The vesicles can be loaded with an ion-sensitive fluorophore to directly measure the initial rates of ion transport across the membranes using spectrofluorometric methods. Using this technique the activity of a K^+-stimulated H^+-ATPase was measured in inner envelope membrane vesicles (Shingles and McCarty 1994). The activity of this enzyme within membrane vesicles was subsequently shown to be equivalent to the pumping of H^+ out of the chloroplast (Shingles and McCarty 1995a). A H^+ gradient across the chloroplast inner envelope stimulates PO_4^{2-}/3PGA and SO_4^{2-}/3PGA exchange (Shingles and McCarty 1995b) and assists in the diffusional movement of NO_2^-, as HNO_2 (Shingles et al. 1997); and HCO_3^-, as CO_2 (Shingles and Moroney 1997). Calcium ion flux across the inner envelope was also stimulated by an electrochemical proton gradient, the driving force being the potential gradient itself (Roh et al. 1998). The spectrofluorometric determination of the initial rate of ion transport gives an indication of the magnitude of the transport processes across the chloroplast inner envelope. In addition the factors which can affect ion transport can also be easily assessed.

Materials and Methods

Chloroplasts were isolated from pea (*Pisum sativum* L. cultivar Laxton's Progress No. 9) by the method of Joy and Mills (1987). Chloroplast inner envelope vesicles were isolated as described by Keegstra and Yousif (1986). Inner envelope vesicles loaded with a specific ion-sensitive fluorophore were prepared by a freeze/thaw technique to produce vesicles of largely inside-out orientation or using a small volume extruder to produce vesicles of largely right side-out orientation (Shingles and McCarty 1995a). The ion-selective fluorophores used in this study include pyranine for H^+, Fura-2 for Ca^{2+}, and Phen Green SK for Fe^{2+}. Fluorescence measurements were collected with an OLIS-modified SLM-SPF-500C spectrofluorometer equipped with an OLIS USA-SF stopped-flow apparatus. Calibration of fluorophore fluorescence to ion transport was determined as described in specific references cited in Table 1.

Results and Discussion

The activity of a H^+- ATPase could be a means of maintaining stromal/cytosolic changes in pH (Berkowitz and Peters 1993, Maury et al. 1981) or be involved in H^+/K^+ exchange across the membrane (Berkowitz and Peters 1993, Huber and Maury 1980, Maury et al. 1981, Wu and Berkowitz 1992). In addition, this H^+- ATPase could set up a gradient equivalent to about 0.5 pH units suggesting that this protein could also function to support the transport of ions across the chloroplast inner envelope.

Cation Transport

Fe^{2+} transport may occur through a putative channel such as the recently identified "iron-regulated transporters" which have homologues in *Arabidopsis* and *Pisum* as well as isoforms with chloroplast targeting sequences (Guerinot 2000). The chloroplast inner envelope shows evidence for an active transporter (Table 1), which appears to have bi-directional activity. The overall activity is quite low compared to other ions studied but this may reflect the low availability of iron within cells due to its' toxic nature. Iron transport activity was stimulated by a potential gradient across the membrane (Table 2) Some of the proteins identified in this class of metal transporters may transport zinc in addition to iron, accounting for the zinc sensitivity of iron transport in pea chloroplast inner envelope preparations (Table 2).

Table 1. Ion transport rates across chloroplast inner envelope vesicles

Ion	Class	Initial Rate	Reference
		(μmol/min/mg prot)	
H^+	pump	0.6	(Shingles and McCarty 1994)
Fe^{2+}	channel	0.007	manuscript submitted
Ca^{2+}	channel	9	(Roh et al. 1998)
SO_4^{2-}/3PGA	exchanger	4	(Shingles and McCarty 1995b)
PO_4^{2-}/3PGA	exchanger	9	(Shingles and McCarty 1995b)
Glycolate/glycerate	exchanger	25	(Young and McCarty 1993)
NO_2^-/HNO_2	diffusion	130	(Shingles et al. 1996)
HCO_3^-/CO_2	diffusion	548	(Shingles and Moroney 1997)

Calcium movement across the chloroplast inner envelope was also stimulated by the potential component of an inwardly directed pH gradient (Roh et al. 1998) and was inhibited by known calcium channel blockers (Table 2) indicating that Ca^{2+} may move through an electrogenic channel. Ca^{2+} transport was favored for movement into right side-out membrane vesicles although some transport could also occur out of the membranes (Fig. 1). While the rate of calcium movement can occur at an order of magnitude higher than the rate of proton pumping (Table 1) there is no evidence for co-transport of a proton with Ca^{2+} transport, therefore the pH gradient should not be dissipated. The rate of calcium transport is over 1000 times greater than the rate of iron transport indicating a high flux rate, important for a signaling molecule. Free calcium levels within the chloroplast are reported to rise from a basal

level of 150 nM to a peak of 5-10 μM upon transition from light to dark (Johnson et al. 1995). Our results indicate that under energetic (light) conditions the chloroplast may be actively taking up calcium from the cytosol.

Table 2. Effectors and inhibitors of ion transport across inner envelope vesicles

Ion	Effector	Inhibitor
H^+	ATP, K^+	gramicidin, vanadate
Ca^{2+}	potential gradient	verapamil, ruthenium red
Fe^{2+}	potential gradient	zinc, cadmium, manganese
SO_4^{2-}/3PGA	pH gradient	2PGA
PO_4^{2-}/3PGA	pH gradient	2PGA
NO_2^-/HNO_2	pH gradient	protonophores
HCO_3^-/CO_2	pH gradient carbonic anhydrase	sulfonamides
glycolate/glycerate	pH gradient	mandelate

Anion Transport

Under physiological conditions the exchange of 3-phosphoglycerate (3PGA) for phosphate occurs with the transfer of a H^+ across the chloroplast inner envelope (Flugge et al. 1983). Proton-linked SO_4^{2-}/3PGA and PO_4^{2-}/3PGA exchanges were both of similar magnitude (Table 1) and inhibited by 2PGA (Table 2) indicating that SO_4^{2-} may be transported on the phosphate translocator. The glycolate/glycerate transporter has much higher rates and different inhibitor sensitivities than the phosphate translocator suggesting that this transport may occur on a different protein than the phosphate translocator. Maximal rates of these transport activities occurred at higher rates than the measured H^+-ATPase activity (Table 1). Therefore the H^+-ATPase may support proton exchange type activities at low concentrations of substrates.

NO_2^- can associate with H^+ to form HNO_2 which can cross the inner envelope membrane at rates clearly sufficient to support the rate of nitrite reductase activity measured within chloroplasts (Shingles et al. 1996). HCO_3^- does not appear to readily cross the inner envelope of pea chloroplasts directly. However an inwardly directed pH gradient promotes the conversion of HCO_3^- to CO_2 and then CO_2 readily crosses the membrane. Inside the membrane vesicles, which are kept at a higher pH of 8.0 (to simulate the pH of the chloroplast stroma), CO_2 is converted back to HCO_3^-. This interconversion between HCO_3^- and CO_2 is greatly stimulated by the presence of carbonic anhydrase on both sides of the membrane (Shingles and Moroney 1997).

All of the anions investigated in this study result in intravesicular protonation. Both HNO_2 and CO_2 cross the inner envelope at high rates. In our isolated membrane system the proton ATPase rate would be insufficient to support such high rates of transport. Therefore it is likely that the chloroplast stroma would provide an effective means of buffering against any pH changes within the chloroplast. This buffering would enable the chloroplast to maintain an inwardly directed pH (and potential) difference which could support the transport of both cations and anions.

Abbreviations: 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate

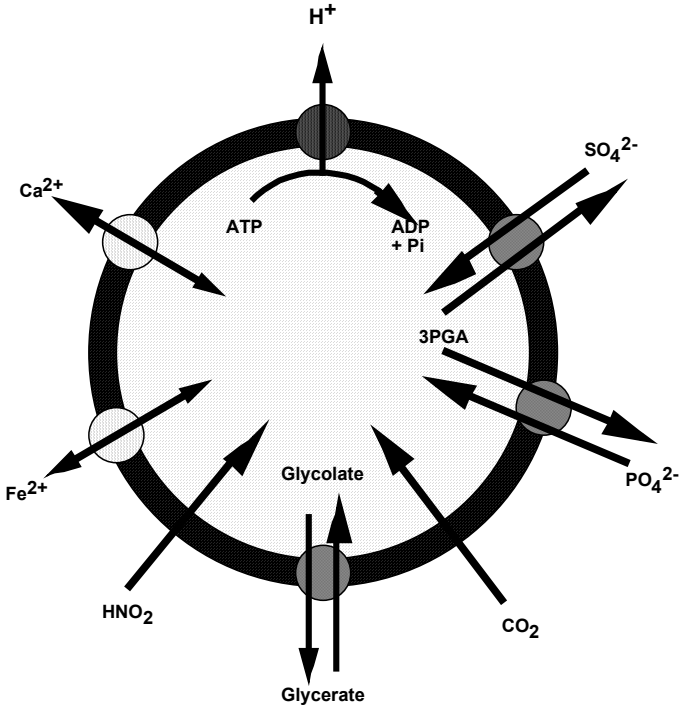

Fig. 1. Proton-linked ion transport across chloroplast inner envelope vesicles

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