Plant Physiol. (1996) 112: 1375–1381

Nitrite Transport in Chloroplast Inner Envelope Vesicles[1]

I. Direct Measurement of Proton-Linked Transport

Richard Shingles, Michael H. Roh, and Richard E. McCarty*

Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218–2685

Chloroplast inner envelope membrane vesicles that are loaded with the pH-sensitive fluorophore, pyranine, show rapid internal acidification when nitrite is added. Acidification is dependent upon ΔpH, with the inside of vesicles being alkaline with respect to the outside. The rate of vesicle acidification was directly proportional to the concentration of nitrite that was added and the imposed pH difference across the membrane. In contrast, added nitrate had no effect on vesicle acidification. Nitrite also caused acidification of asolectin vesicles. The extent of vesicle acidification is dependent on the internal volume of vesicles. Inner envelope and asolectin vesicles that were prepared by extrusion were approximately the same size, allowing them to be compared when the final extent of acidification, measured after the pH gradient had collapsed, was similar. The rate of nitrite-dependent acidification was similar in these two preparations at any single nitrite concentration. These results indicate that nitrite movement occurs by rapid diffusion across membranes as nitrous acid, and this movement is dependent on a proton gradient across the lipid bilayer. Under conditions approximating those in vivo , the rate of diffusion of nitrous acid far exceeds that of nitrite reduction within chloroplasts.

Nitrite reductase converts NO_2^- to NH_4^+. It uses Fd as a reductant (Joy and Hageman, 1966) and is localized in the chloroplast or root plastid (Dalling et al., 1972a, 1972b). Anderson and Done (1978) demonstrated that NO_2^- enters the chloroplast, where it is first reduced to NH_4^+ and then assimilated into Gln. The uptake of NO_2^- was shown to proceed at a much greater rate in the light than in the dark (Brunswick and Cresswell, 1988a), and these authors suggested that the uptake of NO_2^- into the chloroplast could be limiting for NO_2^- reductase activity.

It has been argued that NO_2^- transport across the chloroplast inner envelope occurs by the transport of the protonated form of NO_2^-, HNO_2 (Heber and Purczeld, 1977; Kaiser and Heber, 1983), or by the uptake of the NO_2^- ion (Heber and Purczeld, 1977; Brunswick and Cresswell, 1988a, 1988b). Many of the earlier studies were based on the indirect methods of analysis, including the effect of NO_2^- on CO_2 fixation, O_2 evolution, osmotic swelling, and stromal pH (Purczeld et al., 1978; Enser and Heber, 1980; Kaiser and Heber, 1983). Later studies that used isolated chloroplasts to measure the disappearance of NO_2^- from the bathing medium indicated that NO_2^- uptake was saturable and sensitive to some protein modifiers (Brunswick and Cresswell, 1988a, 1988b), suggesting that there may be a protein transporter for NO_2^-.

Membrane vesicles have been shown to be competent for transport studies (Sze, 1985). Proton movements across phospholipid membranes have been measured fluorometrically using pyranine-loaded vesicles (Biegel and Gould, 1981). These authors demonstrated that asolectin vesicles do maintain a pH gradient over a period of time. The stopped-flow method utilized by Biegel and Gould (1981) allows internal pH changes to be measured instantaneously as protons cross the membrane, with measurement times of less than 2 ms.

Chloroplast inner envelope membrane vesicles that have been loaded with pyranine have also been used to measure the proton flux that is associated with H^+-ATPase activity (Shingles and McCarty, 1994). The function of a H^+-ATPase on the chloroplast inner envelope may be to generate a gradient for proton-linked transport activity (Shingles and McCarty, 1996). Proton-linked transport has been measured in inner envelope vesicles for glycolate/glycerate transport, phosphoglycerate/phosphate exchange, and phosphoglycerate/sulfate exchange (Flugge et al., 1983; Howitz and McCarty, 1988; Young and McCarty, 1993; Shingles and McCarty, 1996). Chloroplast inner envelope vesicles have been shown to have a largely right-side out orientation when prepared by extrusion (Shingles and McCarty, 1995). In such vesicles the movement of NO_2^- by a proton-linked mechanism would result in the acidification of the vesicle interior in a manner resembling that for transport across the inner envelope of intact chloroplasts.

Proton-linked NO_2^- transport across chloroplast inner envelope membrane vesicles could occur by one or a combination of three possible mechanisms. The first is by simple diffusion of HNO_2 across the membranes followed by dissociation inside the vesicles. NO_2^- is in equilibrium with the undissociated acid (HNO_2) both inside and outside of the vesicles (pKa = 3.29 at 25°C).

[1] This work was supported by U.S. Department of Energy grant no. DE-FG02–92ER 200 280.

* Corresponding author; e-mail rem1@jhu.edu; fax 1–410–516–5213.

Abbreviations: Chl, chlorophyll; F_{is}, fluorescence at a pH-insensitive wavelength; F_s, fluorescence at a pH-sensitive wavelength; NEM, *N*-ethylmaleimide; pyranine, 8-hydroxypyrene-1,3,6-trisulfonate.

$$\frac{[NO_2^-]_o[H^+]_o}{[HNO_2]_o} = \frac{[NO_2^-]_i[H^+]_i}{[HNO_2]_i} \quad (1)$$

When the NO_2^- salt is added to these vesicle suspensions, under conditions in which the inside of the vesicles is basic with respect to the outside, HNO_2 will penetrate the membrane and dissociate inside the vesicles, decreasing the pH inside the vesicles. If the rate of NO_2^- transport is very slow relative to that of HNO_2, it follows that when the HNO_2 has equilibrated that:

$$\frac{[NO_2^-]_i}{[NO_2^-]_o} = \frac{[H^+]_o}{[H^+]_i} \quad (2)$$

A second route for proton-linked NO_2^- transport may involve transport of the NO_2^- ion by a symport mechanism with a proton in a process that would be electrically neutral. However, within the membrane vesicles both the NO_2^- ion and the proton would be released, resulting in the protonation of the interior of the membrane vesicles.

A third route for proton-linked NO_2^- transport may be transport of the NO_2^- anion by an antiport mechanism with hydroxide. Acidification of the membrane vesicles would occur as the hydroxyl ions were exported in exchange for NO_2^-.

In this study we used stopped-flow fluorometry to measure proton-linked transport of NO_2^- across chloroplast inner envelope vesicles. This method allows transport activity to be measured directly and can be used to determine the initial rate of proton-linked NO_2^- transport (Young, 1992). The results will be discussed in terms of the species of nitrogen transported, the mechanism of NO_2^- transport, and the implications for providing NO_2^- to the chloroplast for nitrite reductase activity to occur.

MATERIALS AND METHODS

Reagents

Pyranine and *p*-xylene bispyridinium dibromide were purchased from Molecular Probes (Eugene, OR). All other reagents were of the highest grades commercially available.

Plant Material

Pea plants (*Pisum sativum* L. cv Laxton's Progress No. 9) were grown from seeds for 16 to 18 d in vermiculite in a controlled environment growth cabinet (Revco, Asheville, NC) set for 16-h day (24°C)/8-h night (20°C) periods.

Membrane Isolation

Chloroplasts were isolated essentially as described by Joy and Mills (1987). Inner envelope vesicles were prepared according to the method of Keegstra and Yousif (1986). Frozen, intact chloroplasts, equivalent to between 80 and 120 mg of Chl, were thawed at room temperature, refrozen at −20°C, and thawed again. Chloroplast rupture was facilitated by gentle homogenization in a glass Teflon homogenizer. The homogenate was centrifuged at 3,150*g* for 15 min. The supernatants were collected and centrifuged at 27,000*g* for 90 min. Pellets were resuspended in 0.2 M Suc and placed on top of a 0.45/0.80/1.0 M Suc step gradient and centrifuged at 105,000*g* for 18 h. Inner envelope membranes were recovered from the 0.80/1.0 M interface. The yield of purified inner envelopes was approximately 1 mg of protein from isolated chloroplasts equivalent to 100 mg of Chl. All of the above operations were performed at 4°C. Vesicles were subsequently stored under liquid nitrogen.

Vesicle Preparations

Membrane vesicles were prepared using a hand-held, small-volume (0.5–2.5 mL) extrusion apparatus that was similar to the one described by MacDonald et al. (1991) in a buffer containing 5 mM pyranine, 0.1 mM K-Hepes (pH 8.0), 5 mM $MgCl_2$, and 50 mM KCl (Shingles and McCarty, 1995). Typically, 2.0 mL of a membrane suspension that contained about 1 mg of protein of inner envelopes, or 20 mg of asolectin was passed through the extrusion apparatus with a polycarbonate (100-nm pore size) filter in place. From pyranine-loading experiments it was determined that 9 to 11 passes through the membrane resulted in optimal loading (trapped volume of pyranine) in vesicles. An odd number of passes was performed to ensure that all of the collected vesicles had passed through the polycarbonate filter. The vesicle preparation was then passed through a 1.6 × 10 cm Sephadex G-50 column equilibrated with 10 mM K-Hepes (pH 8.0), 5 mM $MgCl_2$, and 50 mM KCl at 4°C to remove external pyranine, and the eluant was diluted to 10.5 mL with the same buffer. The vesicle suspension was allowed to equilibrate for 2 h at 4°C before use.

Size Distribution of Vesicles

Size distribution was determined by quasi-elastic light-scattering analysis (Shingles and McCarty, 1995). The system utilized a Malvern system 4700C submicron particle analyzer (Malvern, UK) with a 5-mW Melles Griot He-Ne laser at an excitation wavelength of 632.8 nm. The size distribution and mean diameters of vesicle suspensions were determined by digital autocorrelation.

Fluorescence Measurements

An Olis modified SLM-SPF-500C spectrofluorometer and an Olis USA-SF stopped-flow apparatus were used (Bogart, GA). Pyranine fluorescence emission was monitored at 512 nm with excitation at 466 nm (F_s) and 418 nm (F_{is} inner envelopes) or 413 nm (F_{is} asolectin). All slits were set at 10 nm with a cutoff filter (LP47, Oriel, Stamford, CT) placed over the entrance to the emission monochromator. Chamber A contained 2.0 mL of vesicle suspension at pH 8.0 plus 5 mM *p*-xylene bispyridinium dibromide to quench the fluorescence of residual external pyranine. Chamber B contained 2.0 mL of buffer at a predetermined pH and the sodium salt of NO_2^- when utilized. Mixing of samples was achieved by a nitrogen-driven piston at 80 p.s.i. All measurements were taken at 25°C.

Data Reduction and Handling

Intravesicular pH was determined from the F_s/F_{is} ratio as previously described (Young and McCarty, 1993; Shingles and McCarty, 1994). Essentially, a calibration curve is generated by adding small aliquots of HCl to the vesicle suspension, followed by measuring the relative fluorescence at F_s 466 and F_{is} 418 (inner envelopes) or F_{is} 413 (asolectin) to determine the F_s/F_{is} ratio, followed by a comparison with the final pH values that were measured with a pH electrode. The data fit the Henderson-Hasselbach equation.

Curve fitting was carried out using the graphing program Kaleidagraph (Synergy Software, Reading, PA). Data were fit to the single exponential equation: $Y = m0*e^{-m1*x}$, where $m0$ = the extent of the pH change (ΔpH) and $m1$ = rate constant (k). Initial rates of vesicle acidification were determined from the relationship $v_i = k*\Delta pH$. The correlation of the fits was greater than 0.95 as determined by the least squares method.

RESULTS

NO_2^--Dependent Vesicle Acidification

The fluorescence of pyranine, when loaded inside membrane vesicles, can be used to determine the internal pH. Excitation at a wavelength at which pyranine fluorescence is pH sensitive (F_s = 466 nm) allows the changes in vesicle acidification to be determined. Dividing these measurements with those collected at a pH-insensitive wavelength (F_{is} = 418 nm) accounts for any small changes in the ionic strength of the solution or osmotic effects due to NO_2^- addition. In control experiments without vesicles and no imposed pH difference, NO_2^- had no effect on pyranine fluorescence measured at either the F_s or F_{is}. However, a decrease in pH resulted in a decrease in the F_s/F_{is} ratio. In the stopped-flow apparatus used in this study membrane vesicles at pH 8.0 were mixed with a buffer at pH 7.0 to impose a pH gradient across the membrane. Chloroplasts maintain a gradient (alkaline in the stroma) of a similar magnitude in the light. In control experiments in which membrane vesicles were mixed with buffer alone there was a slow decrease in pyranine fluorescence, taking approximately 10 min to come to equilibrium (Fig. 1). When NO_2^- was added the decrease in pyranine fluorescence was biphasic, with an initial rapid rate of vesicle acidification within the first 0.5 s, followed by a slower rate during the next 10 min. The slow rate of acidification eventually reached the same equilibrium level as in control experiments.

NO_2^- inhibits CO_2-dependent O_2 evolution, whereas nitrate has no effect (Heber and Purczeld, 1977; Purczeld et al., 1978). The inhibition was attributed to acidification of the chloroplast stroma by NO_2^- but not by nitrate. When 5 mM NO_2^- was added to inner envelope vesicles a rapid pH decrease of approximately 0.5 pH unit was observed within the first 0.5 s (Fig. 2). In contrast, added nitrate (5 mM) caused little pH change. The nitrate results were similar to the control in which vesicles were mixed with pH 7.0 buffer in the absence of nitrate or NO_2^-.

Effect of pH on NO_2^--Stimulated Vesicle Acidification

Purczeld et al. (1978) demonstrated that NO_2^- can cross the chloroplast inner envelope as nitrous acid in a pH-dependent manner. Transport of the NO_2^- anion on a carrier by H^+ symport or OH^- antiport would be expected to have a pH optimum in the physiological pH range. To investigate the effect of the external pH on NO_2^- movement across the inner envelope, a series of experiments was performed by varying the external pH while maintaining the intravesicular pH at 8.0. The initial rates were determined during the first 0.5 s after mixing. The results presented in Figure 3 show a clear dependence of vesicle acidification with added NO_2^- on a pH gradient across the membrane vesicles. Essentially no vesicle acidification occurred at equivalent pH inside and outside the vesicles. When the external pH was decreased the initial rate of proton-linked vesicle acidification increased.

In these experiments changing the external pH also changes the proton concentration difference (ΔpH) across the membrane. This changes the potential driving force of the reaction if NO_2 is co-transported with H^+ and would not be inconsistent with a symport-mediated process. However, the results shown in Figure 3 parallel the expected increase in HNO_2 concentration as the pH was lowered (data not shown). Inner envelope vesicles prepared by extrusion are largely right-side out in orientation

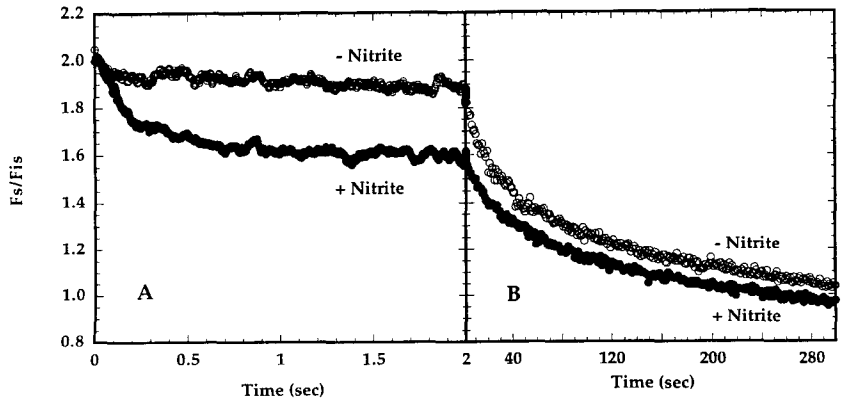

Figure 1. NO_2^--dependent acidification of chloroplast inner envelope vesicles. Pea chloroplast inner envelope vesicles were loaded with 5 mM pyranine as described in "Materials and Methods." The vesicle suspension (100 μg protein/mL) at pH 8.0 was mixed with buffer ± 5 mM sodium nitrite at pH 7.0. Fluorescence emission at 512 nm was monitored at excitation wavelengths of 466 nm (F_s) and 418 nm (F_{is}). Two rates were collected with 600 points in the first 2 s (A) followed by 298 points over the next 298 s (B).

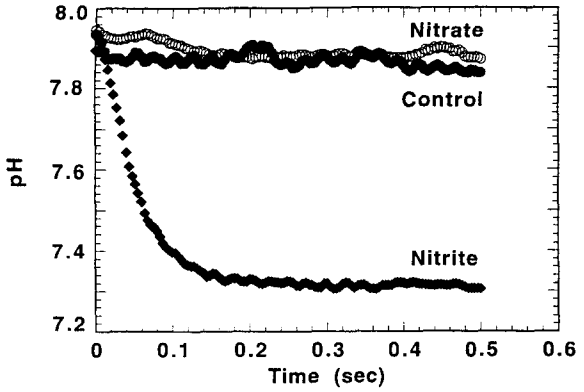

Figure 2. NO_2^- and nitrate effects on intravesicular pH changes. Experimental details are the same as described in Figure 1. The F_s/F_{is} data were converted to pH as described by Shingles and McCarty (1994).

(Shingles and McCarty, 1995). For a comparison, inner envelope vesicles were prepared using a freeze-thaw method that produces vesicles that are largely inside out. In these preparations proton-linked NO_2^- transport was also rapid and dependent on a pH gradient (data not shown).

NO_2^--Dependent Acidification in Asolectin Vesicles

The results in Figure 3 suggest that proton-linked NO_2^- transport may occur as a result of HNO_2 diffusion across the membrane. To investigate this diffusive component further, comparative studies were conducted using inner envelope and asolectin vesicles. Asolectin, a protein-free lipid mixture, forms vesicles that allow the investigation of the diffusive component of NO_2^- transport. To compare asolectin vesicles with inner envelope vesicles it is necessary to have the internal vesicle volumes approximately equivalent. Two factors determine the vesicle volumes

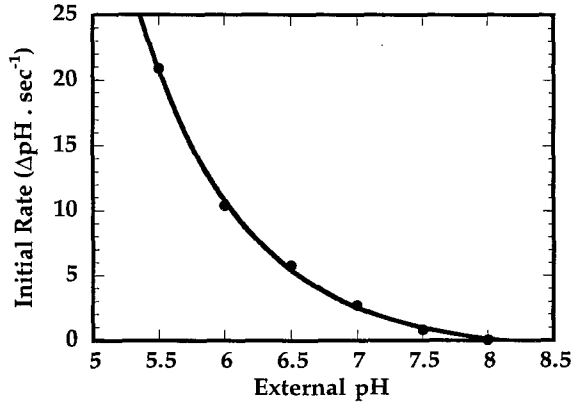

Figure 3. Effect of external pH on the initial rate of NO_2^--dependent vesicle acidification. Pyranine-loaded inner envelope vesicles (pH inside = 8.0) were mixed with buffers at different pH values plus 1 mM NO_2^-. The initial rate of vesicle acidification was determined over the first 0.5 s after mixing using the equation describing a single exponential decrease.

in these experiments, the size of the vesicles and their concentration.

Laser light-scattering measurements give a good indication of the size of membrane vesicles (Shingles and McCarty, 1995). When these measurements were performed on inner envelope and asolectin vesicles that had been prepared by extrusion, the results indicated that the size distribution of vesicles was similar (Fig. 4). Asolectin vesicles had a mean diameter of 95 nm and inner envelope vesicles were only slightly smaller with a mean diameter of 90 nm, a difference of less than 20%.

Within a given set of experiments, such as those represented in Figure 1, the fluorescence decrease comes to a certain equilibrium level after 10 min. This overall difference represents the final extent of the pH change, which is expected to vary with changes in vesicle volume and with the number of vesicles. Within a set of experiments vesicle size was uniform; hence, the extent changes with vesicle concentration. The extent of the pH change can be varied using different dilutions of asolectin vesicles. The initial rate of vesicle acidification, in the presence of 5 mM NO_2^-, was determined at each series of dilutions, giving changes in the extent of the pH change (Fig. 5). A linear relationship exists between the initial rate of vesicle acidification and the extent of the pH change. The rate constant was unaffected by differences in the extent of the pH change. This result is important for a comparison of the results for asolectin and inner envelope vesicles, since it is necessary to begin with preparations that show similar final extents

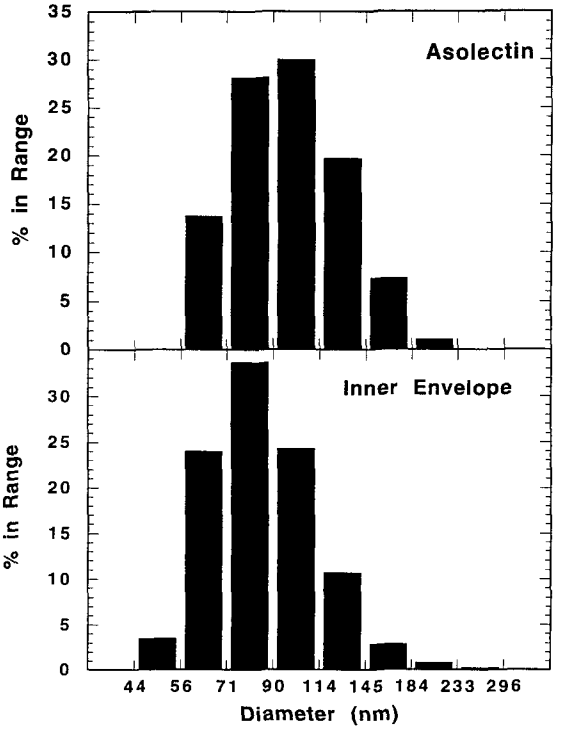

Figure 4. Size distribution of asolectin and inner envelope membrane vesicles. Vesicles were prepared by extrusion through a 100-nm polycarbonate filter, and diameters were measured by quasi-elastic light scattering as described by Shingles and McCarty (1995).

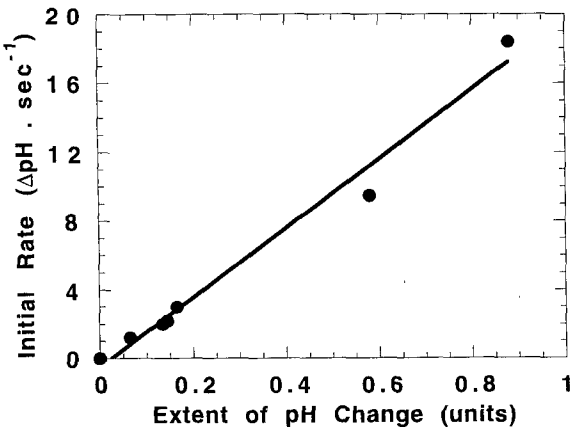

Figure 5. Relationship between the initial rate of NO_2^--dependent vesicle acidification and the extent of the pH change. The extent of the pH change was altered by diluting vesicles with buffer. NO_2^- (5 mM) was added at each dilution, and the initial rate of vesicle acidification was determined over the first 0.5 s.

of ΔpH to compare the rate constants and the initial rates of transport.

Using an asolectin preparation that had the same final extent of pH change over 10 min as an inner envelope preparation, we determined the rate constant, extent of pH change, and the initial velocity of proton-linked NO_2^- transport at different concentrations of NO_2^- (Table I). The initial rate for proton-linked movement across membranes is the product of the rate constant and the extent of the ΔpH. In asolectin and inner envelope controls in which no NO_2^- was added, the rate constant and extent of pH change were the same, resulting in initial rates that were identical. In inner envelope vesicles both the rate constant and the extent of the pH change increased as NO_2^- concentration increased (Table I). This results in the initial rate for proton-linked NO_2^- movement increasing with increased NO_2^- concentration. Similar results were obtained with asolectin vesicles, where both the rate constant and the extent of the pH change increased with increasing NO_2^- concentrations. In general, there is a linear relationship between the rate of proton-linked NO_2^- transport and the NO_2^- concentration. The initial rates of proton-linked NO_2^- transport in asolectin and inner envelopes are similar at each NO_2^- concentration tested, suggesting that proton-linked NO_2^- transport occurs across the membrane vesicles by the same mechanism.

DISCUSSION

NO_2^- transport into intact chloroplasts has been measured using a variety of indirect assays, including the effect of NO_2^- on CO_2 fixation, O_2 evolution, and stromal pH (Purczeld et al., 1978; Enser and Heber, 1980; Kaiser and Heber, 1983). The NO_2^--induced changes in these assays typically occurred during the course of several minutes. Osmotic swelling of intact chloroplasts has proven to be a better way to measure rapid changes that are induced by the addition of NO_2^-. Rates of NO_2^- influx were determined to be approximately 1000 μeq mg^{-1} Chl h^{-1} with 100 mM added NO_2^- (Heber and Purczeld, 1977). Unfortunately, the time resolution of this assay is too low to make accurate determinations of the initial rate for NO_2^- transport, especially at physiological concentrations.

The use of chloroplast inner envelope membrane vesicles that are loaded with the pH-dependent fluorophore, pyranine, enables proton-linked activities to be measured, which, when used with stopped-flow spectrofluorometry, allows for measurement of small pH changes with a resolution of less than 2 ms. NO_2^--dependent vesicle acidification was rapid, coming to equilibrium within 0.5 s (Figs. 1A and 2). The initial rate (V_i) of transport at 0.5 mM NO_2^- in this study was determined to be 0.9 pH unit s^{-1} (Table I). In a previous study the internal buffering capacity (β_{in}) of inner envelope vesicles was determined to be 240 nmol H^+ pH^{-1} mg^{-1} protein, and there are 100 μg inner envelope protein mg^{-1} Chl in intact chloroplasts (Young and McCarty, 1993). The rate of NO_2^--dependent vesicle acidification is the product of V_i and β_{in} and was determined to be 78 μmol H^+ h^{-1} mg^{-1} Chl at a NO_2^- concentration of 0.5 mM and 25°C.

The assimilation of nitrate involves the conversion of nitrate to NO_2^- by nitrate reductase, followed by the transport of NO_2^- across the chloroplast inner envelope. Within the chloroplast stroma nitrite reductase reduces NO_2^- to NH_4^+, which is further incorporated into amino acids (Anderson and Done, 1978). Nitrite reductase activity has been measured in isolated chloroplasts from spinach and pea preparations and shown to range from 1 to 12 μmol h^{-1} mg^{-1} Chl at 0.33 to 0.5 mM NO_2^- (Magalhaes et al.,

Table I. *Comparison of the initial rates for proton-linked NO_2^- transport between chloroplast inner envelope membranes and asolectin vesicles*

Preparations of asolectin and inner envelope vesicles having similar final extent of pH changes were compared at several NO_2^- concentrations. The rate constant (k), extent of pH change (ΔpH), and initial rates (V_i) were determined for a single exponential decrease during the first 0.5 s after NO_2^- addition.

NO_2^+	Inner Envelopes			Asolectin		
	k	Extent	V_i	k	Extent	V_i
mM	s^{-1}	ΔpH	ΔpH s^{-1}	s^{-1}	ΔpH	ΔpH s^{-1}
0	1.14	0.07	0.1	1.14	0.07	0.1
0.5	4.76	0.20	0.9	7.46	0.22	1.6
1.0	10.05	0.28	2.8	11.10	0.25	2.6
2.0	16.30	0.58	9.5	13.71	0.64	8.7
5.0	25.56	0.80	20.4	20.57	0.89	18.3

1974; Anderson and Done, 1978; Brunswick and Cresswell, 1988a). Therefore, the rate of proton-linked NO_2^- transport is approximately 5 to 80 times greater than the measured activity of NO_2^- reductase in chloroplasts. The NO_2^- content in shoot and root tissues of barley plants grown with NO_2^- as the sole nitrogen source has been measured (Siddiqi et al., 1992). If it was assumed that all of the NO_2^- were localized in the cytoplasm, then the NO_2^- concentration of that compartment could be estimated based on the assumption that the cytoplasm occupies approximately 5% of the cell volume (Lee and Ratcliffe, 1983). Using this assumption Siddiqi et al. (1992) estimated the cytoplasmic NO_2^- concentration to be 2.8 mM in roots and 0.16 to 0.2 mM in shoots. Although these levels are 2 to 3 times lower than the concentration of NO_2^- used in this study, the rate of proton-linked NO_2^- movement across the membrane clearly has the capacity to move higher concentrations of NO_2^- across the membrane very rapidly. These data indicate that NO_2^- transport across the inner envelope would not be a limiting step in the overall conversion of nitrate to NH_4^+.

In contrast to NO_2^- transport, there was no measurable acidification as a result of the transport of nitrate across chloroplast inner envelope vesicles (Fig. 2). The proton-linked transport of nitrogenous compounds across the chloroplast envelope is obviously selective for NO_2^- transport over nitrate transport.

The transport of NO_2^- was shown to be light-dependent (Brunswick and Cresswell, 1988a), indicating that a proton gradient across the chloroplast inner envelope aids in the uptake of NO_2^-. Brunswick and Cresswell (1988b) also suggested that there might be a proteinaceous transporter for NO_2^-, based on the inhibitory effect of the protein-modifying agent, NEM, on NO_2^- uptake. This association is tenuous, however, since NEM is membrane permeant and might have effects on other enzymes possibly involved in maintaining the pH gradient across the chloroplast envelope. For instance, the H^+-ATPase activity measured in inner envelope vesicles by Shingles and McCarty (1994) was also inhibited by NEM. This ATPase may be involved in maintaining the pH gradient across the chloroplast inner envelope for proton-linked transport activity (Shingles and McCarty, 1996). In addition to its effects on the H^+-ATPase, NEM can inhibit noncyclic electron transport at the level of Fd-$NADP^+$ oxidoreductase (Mills et al., 1979). This inhibition would also decrease the pH of the stroma, possibly resulting in a decrease in NO_2^- uptake. Our results do not preclude the existence of a high-affinity NO_2^- translocator that does not cause a change in the pH of the vesicle interior. An anion channel, for example, could be present that transports NO_2^-. Brunswick and Cresswell (1988a) suggested the presence of an NO_2^- transporter that operates at an alkaline pH optimum. However, the rate of transport by this mechanism was almost 200 times slower than the rates measured in this study. In view of the high rate of HNO_2 diffusion, there appears to be no need for such a transporter in illuminated chloroplasts.

Several lines of evidence in this study suggest that proton-linked NO_2^- transport activity occurs due to the diffusion of HNO_2 across the inner envelope membrane, followed by its dissociation to H^+ and NO_2^-, as indicated by Equations 1 and 2. First, proton-linked NO_2^- transport was clearly dependent on the external pH (Fig. 3). If the transport of NO_2^- occurred by a symport mechanism translocating H^+ and NO_2^-, one would expect a clear pH optimum for this activity. However, the data in Figure 3 indicate that the initial rate of proton-linked NO_2^- transport parallels the increase in HNO_2 concentration as the exterior pH is lowered. Similar results were seen for both right-side out and inside out inner envelope vesicles, indicating that there was no directional component to proton-linked NO_2^- transport (data not shown). Second, in the assay utilized in this study NEM had no effect on the initial rate of proton-linked NO_2^- transport (data not shown), suggesting that the inhibition observed by Brunswick and Cresswell (1988b) may be attributable to some component of the chloroplast that is involved in maintaining the pH gradient across the inner envelope. Third, the initial rates of proton-linked transport between inner envelope and asolectin vesicles were similar at each of the NO_2^- concentrations tested (Table I). Asolectin vesicles do not contain any transport proteins; therefore, any movement that is measured can be attributed to substrate diffusion across the membrane. Since diffusion of NO_2^- would not be proton-linked, this result indicates that the proton-linked transport activity measured in asolectin and inner envelope membrane vesicles occurs by a similar mechanism, the rapid diffusion of HNO_2 across the membrane.

In this study high rates of proton-linked NO_2^- transport were measured across chloroplast inner envelope membrane vesicles and are attributed to the movement of HNO_2. The rapid movement of HNO_2 and other weak acids across the inner envelope would require some active proton transport from the stroma into the external space (Purczeld et al., 1978). The presence of an H^+-ATPase on the chloroplast inner envelope, which pumps H^+ out of the stroma into the cytosol, may fulfill this role (Shingles and McCarty, 1994, 1995). Maintenance of a pH gradient across this membrane would ensure that significant levels of HNO_2 would continue to cross the chloroplast envelope to supply nitrite reductase with its substrate.

ACKNOWLEDGMENT

We thank Dimeka Patterson for cultivating the peas and preparing the chloroplasts.

Received May 20, 1996; accepted August 6, 1996.

LITERATURE CITED

Anderson JW, Done J (1978) Light-dependent assimilation of nitrite by isolated pea chloroplasts. Plant Physiol **61:** 692–697

Biegel CM, Gould JM (1981) Kinetics of hydrogen ion diffusion across phospholipid vesicle membranes. Biochemistry **20:** 3474–3479

Brunswick P, Cresswell CF (1988a) Nitrite uptake into intact pea chloroplasts. I. Kinetics and relationship with nitrite assimilation. Plant Physiol **86:** 378–383

Brunswick P, Cresswell CF (1988b) Nitrite uptake into intact pea chloroplasts. II. Influence of electron transport regulators, uncouplers, ATPase and anion uptake inhibitors and protein binding reagents. Plant Physiol **86:** 384–389

Dalling MJ, Tolbert NE, Hageman RH (1972a) Intracellular localization of nitrate reductase and nitrite reductase. I. Spinach and tobacco leaves. Biochim Biophys Acta **283:** 505–512

Dalling MJ, Tolbert NE, Hagemann RH (1972b) Intracellular localization of nitrate reductase and nitrite reductase. I. Wheat roots. Biochim Biophys Acta **283:** 513–519

Enser U, Heber U (1980) Metabolic regulation by pH gradients: inhibition of photosynthesis by indirect proton transfer across the chloroplast envelope. Biochim Biophys Acta **592:** 577–591

Flugge UI, Gerber J, Heldt HW (1983) Regulation of the reconstituted chloroplast phosphate translocator by a H^+ gradient. Biochim Biophys Acta **725:** 229–237

Heber U, Purczeld P (1977) Substrate and product fluxes across the chloroplast envelope during bicarbonate and nitrite reduction. *In* DO Hall, J Coombs, TW Goodwin, eds, Proceedings of the Fourth International Congress on Photosynthesis. The Biochemical Society, London, pp 107–118

Howitz KT, McCarty RE (1988) Measurement of proton-linked transport activities in pyranine loaded chloroplast inner envelope vesicles. Plant Physiol **86:** 999–1001

Joy KW, Hageman RH (1966) The purification and properties of nitrite reductase from higher plants, and its dependence on ferredoxin. Biochem J **100:** 263–273

Joy KW, Mills WR (1987) Purification of chloroplasts using silica sols. Methods Enzymol **148:** 179–188

Kaiser G, Heber U (1983) Photosynthesis of leaf cell protoplasts and permeability of the plasmalemma to some solutes. Planta **157:** 462–470

Keegstra K, Yousif AE (1986) Isolation and characterization of chloroplast envelope membranes. Methods Enzymol **118:** 316–325

Lee RB, Ratcliffe RG (1983) Phosphorus nutrition and the intracellular distribution of inorganic phosphate in pea root tips: quantitative study using ^{31}P-NMR. J Exp Bot **34:** 1222–1244

MacDonald RC, MacDonald RI, Menco BPM, Takeshita K, Subbarao NK, Hu L (1991) Small volume extrusion apparatus for preparation of large unilamellar vesicles. Biochim Biophys Acta **1061:** 297–303

Magalhaes AC, Neyra CA, Hageman RH (1974) Nitrite assimilation and amino-nitrogen synthesis in isolated spinach chloroplasts. Plant Physiol **53:** 411–415

Mills JD, Crowther D, Slovacek RE, Hind G, McCarty RE (1979) Electron transport pathways in spinach chloroplasts: reduction of the primary acceptor of photosystem II by reduced nicotinamide adenine dinucleotide phosphate in the dark. Biochim Biophys Acta **547:** 127–137

Purczeld P, Chen CJ, Portis AR Jr, Heldt HW, Heber U (1978) The mechanism of the control of carbon fixation by the pH in the chloroplast stroma: studies with nitrite-mediated proton transfer across the envelope. Biochim Biophys Acta **501:** 488–498

Shingles R, McCarty RE (1994) Direct measurement of ATP-dependent proton concentration changes and characterization of a K^+-stimulated ATPase in pea chloroplast inner envelope vesicles. Plant Physiol **106:** 731–737

Shingles R, McCarty RE (1995) Production of membrane vesicles by extrusion: size distribution, enzyme activity and orientation of plasma membrane and chloroplast inner envelope membrane vesicles. Anal Biochem **229:** 92–98

Shingles R, McCarty RE (1996) Proton-linked transport activity across chloroplast inner envelope vesicles. *In* P Mathis, ed, Photosynthesis: From Light to Biosphere. Kluwer Academic, Dordrecht, The Netherlands, pp 75–78

Siddiqi MY, King BJ, Glass ADM (1992) Effects of nitrite, chlorate and chlorite on nitrate uptake and nitrate reductase activity. Plant Physiol **100:** 644–650

Sze H (1985) H^+-translocating ATPases: advances using membrane vesicles. Annu Rev Plant Physiol **36:** 175–208

Young XK (1992) Metabolite transport across the chloroplast envelope. PhD thesis. Cornell University, Ithaca, NY

Young XK, McCarty RE (1993) Assay of proton-coupled glycolate and D-glycerate transport into chloroplast inner envelope membrane vesicles by stopped-flow fluorescence. Plant Physiol **101:** 793–799