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K^+-conducting ion channel of the chloroplast inner envelope: Functional reconstitution into liposomes

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ABSTRACT **Potassium flux between the chloroplast stroma and cytoplasm is known to be indirectly linked to H^+ countertransport and, hence, stromal pH and photosynthetic capacity. The specific molecular mechanism that facilitates K^+ flux across the chloroplast envelope is not known and has been a source of controversy for well over a decade. The objective of this study was to elucidate the nature of this envelope protein. To this end, solubilized protein in detergent extracts of purified chloroplast inner envelope vesicles was reconstituted into artificial liposomes, and cation fluxes into these proteoliposomes were measured. Results of inhibitor studies and counterflux experiments indicated that a K^+-conducting ion channel was solubilized and functionally reconstituted into the proteoliposomes. This transport protein may be a nonspecific monovalent cation channel. This report represents a direct demonstration of ion channel activity associated with the limiting (inner) membrane of the chloroplast envelope.**

Photosynthesis is critically dependent on the maintenance of high stromal pH in the illuminated chloroplast (1) due to the high pH optima of several photosynthetic carbon reduction cycle enzymes. This important regulatory phenomenon necessitates development of a pH gradient between the illuminated stroma (at pH $\approx$ 8) and both the thylakoid lumen (pH $<$ 6) and the external medium (i.e., cytosol, at pH 7) *in situ*. For well over a decade it has been known that H^+ flux across the chloroplast envelope is indirectly linked to K^+ countercurrents, which likely occur through a separate (well-regulated) uniport pathway (2). Inhibitor studies with intact isolated chloroplasts further suggest that trans-envelope K^+ flux may occur through a putative ion channel (2–4).

Characterization of this K^+ transport pathway, however, has not been possible because of the nature of the experimental system (i.e., the intact chloroplast) used in previous work. Definitive conclusions about this putative ion channel await the development of an experimental system—i.e., extraction of the protein from its native membrane, maintenance of its *functional* integrity, reconstitution into a homogenous membrane system (e.g., artificial liposomes), and development of a transport assay sensitive enough to measure the specific activity of the protein. These objectives were the primary focus of the experiments described in this report. The significance of the work is underscored by the following: (*i*) the existence of ion channels in the inner membrane of the chloroplast envelope is demonstrated—the biophysical nature of the envelope cation transporter has been the subject of speculation for well over a decade (5, 6); and (*ii*) relatively little is known about K^+ channels in general—molecular characterization of this class of proteins is just beginning and purification of a functional K^+ channel from any (plant or animal) source has not yet been reported (7).

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MATERIALS AND METHODS

Preparation of Membrane Vesicles, Protein Extraction, and Reconstitution. Field-grown spinach (*Spinacia oleracea* var. Melody) plants (10–15 kg) were used to prepare large quantities (100–200 mg of chlorophyll) of intact chloroplasts, which were then ruptured by freeze/thaw cycles as described (8). Inner chloroplast envelope membrane vesicles (with no detectable thylakoid membrane contamination) were then purified by using a discontinuous sucrose density gradient (8). Chloroplast inner envelope vesicles (typically 5–10 mg of protein) were suspended in envelope medium (0.2 M sucrose/10 mM Tricine NaOH, pH 7.5/2 mM Na_2EDTA/2 mM dithiothreitol) at 1.25 mg of protein per ml and stored at −80°C. Aliquots (800 μl) of membrane vesicles in envelope medium minus sucrose were brought to 1% (wt/vol) sodium cholate (prepared from cholic acid purified according to ref. 9) and 0.2% asolectin with 100-μl additions from stocks (made up as described in ref. 11), left on ice for 20 min, and pelleted by centrifugation (10 min) in a Beckman Airfuge at 30 psi (1 psi = 6.9 kPa) (178,000 × *g*). Detergent-extracted protein (≈40% of total) recovered in the supernatant can be stored at −80°C for months. Protocols for reconstitution of detergent-extracted protein into enlarged liposomes followed those described by Howitz and McCarty (11) except that column buffer was Hepes/Tris, pH 8.0, and the sonication cycle was five 2-s bursts. For counterflux experiments, the protein/lipid column eluent was diluted 9:1 with 1 M NaCl or KCl in column buffer.

Transport Assays. The key technical aspect of this work was the development of an assay sensitive enough to measure channel-mediated cation flux in a heterogenous population of proteoliposomes. The standard microfiltration assay (e.g., as in ref. 11) was not adequate for our work. We increased the sensitivity by decreasing filter area ≈50-fold and using two radioisotopes (one impermeant) to allow for background correction within each sample replicate. Assay tubes were constructed by forcing the cut end (at the 0.1-ml mark) of a 1-ml plastic syringe into a 400-μl microcentrifuge tube, with a 0.5-cm^2 square of 10-μm-mesh nylon net and a 6-mm-diameter microfilter [0.22-μm-pore MSI (Westboro, MA) nitrocellulose; this filter performed better than others] disc sandwiched in between. The nylon net holds the filter disc tightly against the bottom of the syringe tip, which has a 1.6-mm-diameter opening. The filter was prewetted with 10 μl of reaction medium (containing no radioisotope), and 80 μl of reaction medium (containing proteoliposomes and radioisotopes) was pipetted into the syringe tip. Adhesion to the plastic walls keeps the solution away from the filter disc. The assay tube was centrifuged (after 5 min unless otherwise noted) in a Beckman Microfuge B for 15 s. [^{3}H]Mannitol (an impermeant, radiolabeled solute) was included in all reaction

Abbreviations: TEA, tetraethylammonium; TPP^+, tetraphenylphosphonium; TTX, tetrodotoxin.

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media to allow for estimation of the radiolabeled cation retained on the filter but not taken up into the liposomes. Typically, experiments were initiated by adding liposomes (8 μg of protein per tube) in column buffer to an equal volume of reaction solution containing chloride salts of $^{86}Rb^+$ (≈5 μCi/ml; 1 Ci = 37 GBq) or $^{22}Na^+$ (4.5 μCi/ml), [^{3}H]mannitol (5–10 μCi/ml), 20 mM Tricine/Tris, pH 8.0, and additions as noted for each experiment. In some experiments, the internal osmotic volume of the proteoliposomes, in addition to the (external) background volume, was measured by including [^{14}C]mannitol in the incubation solution during the freeze/thaw/sonication cycles.

For counterflux experiments, proteoliposomes that had been preequilibrated with 100 mM NaCl or KCl were added at a 1:9 (vol/vol) ratio to reaction solution containing (final concentration) either 90 mM Tris·HCl and 10 mM NaCl (or KCl), or 100 mM NaCl (or KCl), and radioisotopes as noted above. Upon addition of proteoliposomes (2 μg of protein per tube) to the reaction solution in counterflux experiments, then, the proteoliposomes were subjected to a condition in which $K^+(Na^+)_{in}$ = 100 mM and $K^+(Na^+)_{out}$ = 10 mM, or $K^+(Na^+)_{in}$ = 100 mM and $K^+(Na^+)_{out}$ = 100 mM. After centrifugation, the filter discs were removed from assay tubes, wetted with 50 μl of 10 mM KOH, placed in 7 ml-scintillation vials with 0.5 ml of 1% Triton X-100, and left on a rotary shaker (350 rpm) for 30 min prior to addition of scintillation mixture. For all experiments, data are reported as the means of four replications (±SE) for each treatment.

Reagents. Asolectin was from Associated Concentrates (Woodside, NY); tetraphenylphosphonium (TPP^+) was from Fluka; [^{3}H]mannitol, ^{86}RbCl, and ^{22}NaCl were from New England Nuclear; and nylon nets were from Tetko (Elmsford, NY). All other reagents were from Sigma.

RESULTS AND DISCUSSION

Development and Validation of the Assay System. Preliminary experiments involving the measurement of external (solution external to proteoliposomes) and internal (osmotic space of the proteoliposomes) volumes retained on filter discs (data not shown) indicated that (*i*) the internal volume in the filtrate is minimal (most of the proteoliposomes are retained on the filter during an initial spin); (*ii*) there is no significant time-dependent decrease in internal volume or increase in percent background (i.e., the mannitol is not penetrating into the osmotic volume of the proteoliposomes); and (*iii*) no net internal volume is retained on the filter in the absence of proteoliposomes. These results indicate that protein was reconstituted into tightly sealed proteoliposomes, and the assay system effectively measures ion uptake into the osmotic space of these proteoliposomes.

Due to use of two radioisotopes (facilitating background subtraction within each sample) and a reduction in filter area in contact with label, measurements of cation uptake can be made with this assay system without washing the filters. The effect of washing on cation uptake into proteoliposomes was investigated by ascertaining net K^+ (i.e., $^{86}Rb^+$) in proteoliposomes that had been retained on the filter during a standard transport assay (10 mM external K^+) after a series of washes. Washing was facilitated by the addition of 80 μl of reaction solution (without label) into the upper reservoir of assay tubes after the initial centrifugation. The newly added solution came into contact with the filter only briefly, as it passed into the microcentrifuge tube upon recentrifugation. For one such experiment, a K^+ uptake level of 19.7 ± 1.0 nmol of K^+ per mg of lipid was reduced to 6.0 ± 3.9 and 4.3 ± 0.9, respectively, after one and two washes. It is not surprising that such "instantaneous" washing would reduce cation accumulation into proteoliposomes by 70–80%. Upon exposure to unlabeled solution, liposomes that contain a channel should lose their label faster (by orders of magnitude) than liposomes without a channel. In all likelihood, washing would obscure the ability to observe channel-mediated cation uptake into proteoliposomes.

Documentation of the Presence of a Functional K^+-Conducting Channel in Proteoliposomes. One accepted method of demonstrating that a functional channel has been reconstituted into artificial liposomes is by an enhanced percentage of label taken up into the total proteoliposome population under counterflux conditions (12). Proteoliposomes preequilibrated with a high concentration of a permeant cation are exposed to an external solution that (*i*) has a lowered level of the cation (after dilution), (*ii*) contains a radiolabeled analog of the cation, and (*iii*) is made isotonic by the addition of an impermeant cation (90 mM $Tris^+$). As a consequence of the K^+ (or Na^+) gradient, which occurs upon dilution of the proteoliposomes, an electrical diffusion potential is set up. The magnitude of the potential is much greater in liposomes with a reconstituted channel that is highly conductive to the cation of interest. Even though net flux of the cation is outward in all liposomes, the radiolabeled analog in the exterior solution is selectively equilibrated into liposomes containing a channel. The membrane potential drives radioisotope uptake into the channel-containing liposomes over the course of minutes. In time (5–10 min; ref. 12), the net efflux of the cation out of the proteoliposomes dissipates the K^+ (or Na^+) gradient and the interior negative membrane potential. As a consequence, the radioisotope that had been accumulated now begins to leave the vesicles. Counterflux conditions do reveal the presence of a K^+-conducting channel in at least a portion of the total proteoliposome population (Fig. 1*A*). These results, showing a manyfold increase in the amount of label equilibrating in the proteoliposomes against the imposed concentration gradient, can occur only in the presence of a high-conductance carrier protein such as a functional channel (12).

A second line of evidence documenting the reconstitution of a functional K^+-conducting channel involved the use of the

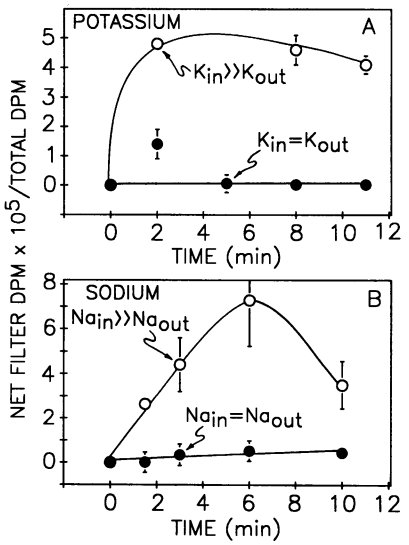

FIG. 1. Demonstration of channel activity in proteoliposomes with reconstituted chloroplast envelope protein by use of counterflux conditions. At time zero, proteoliposomes, which had been prepared in column buffer with 100 mM KCl (*A*) or NaCl (*B*), were diluted into reaction medium that maintained the cation concentration (○) or that diluted the cation to 10 mM and contained 90 mM $Tris^+$ (●). At initiation of the time course of these counterflux experiments, proteoliposomes were exposed to 9.5 μCi of $^{86}Rb^+$ per ml (*A*) or 5.0 μCi of $^{22}Na^+$ per ml (*B*). Cation uptake values are presented as a percentage of total radioisotope in the reaction solution because of changing specific activity during the assay.

K^+ channel blocker tetraethylammonium (TEA) (10). In this case (Table 1), proteoliposomes were incubated in 10 mM K^+. When volume measurements were used to calculate the concentration of K^+ taken up into the interior of the proteoliposomes, it can be seen that the cation essentially equilibrated into the osmotic volume of small proteoliposomes (Exp. 1) and nearly equilibrated into the interior of large proteoliposomes (Exp. 2). TEA substantially reduced K^+ uptake into the proteoliposomes in both experiments. K^+ uptake was reduced in liposomes as compared to proteoliposomes in both experiments, suggesting that reconstitution of the channel increased K^+ conductance across the membrane. Significantly, TEA had no effect on K^+ uptake into liposomes that contained no reconstituted chloroplast envelope protein (Table 1).

Evidence for a Monovalent Cation Channel. Previous work from this (2) and other laboratories (5) has shown that K^+ and Na^+ may be interchangeable in terms of the effects they have on isolated spinach chloroplasts, leading to the speculation that fluxes of these cations across the chloroplast envelope may be facilitated by the same or similar transport proteins (2). This assertion led us to hypothesize that chloroplast inner envelope protein, when reconstituted into artificial liposomes, would also display Na^+-conducting channel activity. Counterflux studies with Na^+-loaded proteoliposomes exposed to $^{22}Na^+$ demonstrated the presence of high Na^+ conductance by a functional channel (Fig. 1*B*). As was the case with K^+ (Fig. 1*A*), counterflux conditions ($Na_{in} \ggg Na_{out}$) led to a transitory accumulation of $^{22}Na^+$, which then began to dissipate after ≈6 min (Fig. 1*B*). Further documentation of Na^+-conducting channel activity in proteoliposomes was afforded by studies with Na^+ channel blockers. As shown in Fig. 2, $^{22}Na^+$ equilibration was inhibited by increasing tetrodotoxin (TTX), a Na^+ channel blocker (13). The K_i deduced from the results shown in Fig. 2 (≈10 μM) is typical of TTX inhibition with some classes of Na^+-conducting channels (14).

In further studies, the effects a range of monovalent cation channel blockers have on Na^+ uptake were examined (Table 2). In addition to an effect by TTX, amiloride (another Na^+ channel blocker; ref. 15) was found to inhibit Na^+ uptake (Table 2). Although lidocaine is widely acknowledged to block Na^+ channels in animal systems (16), work from this laboratory (2, 4) has demonstrated that lidocaine, as well as TEA, blocks K^+ efflux from isolated chloroplasts. Interestingly, $^{22}Na^+$ uptake was sensitive to both lidocaine and the K^+ channel blocker TEA (Table 2). The compound 4-aminopyridine is known to block another class of K^+ channels in animal systems (17). K^+ fluxes across the intact chloroplast are insensitive to 4-aminopyridine (W. H. Wu and G.A.B., unpublished data). This channel blocker was found to have only a minor, statistically insignificant effect on the reconstituted channel(s) (Table 2). Results presented in Table 2 indicate that Na^+-conducting channels derived from the chloroplast envelope are equally sensitive to Na^+ and some K^+ channel blockers. We also found substantial effects of TTX on K^+ (i.e., $^{86}Rb^+$) uptake into proteoliposomes. In two experiments, K^+ uptake of 25.3 ± 2.2 (Exp. 1) and 36.1 ± 3.2 (Exp. 2) nmol of K^+ per mg of lipid was reduced to 18.2 ± 2.7 (26% inhibition) and 26.6 ± 2.3 (28% inhibition) in the presence of 10 μM TTX. These data, along with those shown in Tables 1 and 2, reveal a cross-sensitivity of Na^+ and K^+ conductances to channel blockers. These results suggest the possibility that a single type of chloroplast envelope-derived protein *may* facilitate both Na^+ and K^+ fluxes. This putative chloroplast envelope monovalent cation channel, then, may share some properties with previously characterized monovalent cation channels that conduct Na^+ and K^+ equally well such as the nicotinic acetylcholine receptor (18) or the K^+/Na^+ channel in the sarcoplasmic reticulum of skeletal muscle (19).

The cross-sensitivity of Na^+ and K^+ conductances to a range of monovalent cation channel blockers as reported here is not without precedent. Previous reports have documented the sensitivity of various K^+ channel classes to the Na^+ channel blockers amiloride (20) and lidocaine (21). Even TTX has been found in recent studies (22) to block channels that conduct cations other than Na^+. Treatment of Na^+ channels with Pronase has been found to induce sensitivity of Na^+ conductance to the K^+ channel blocker TEA while still

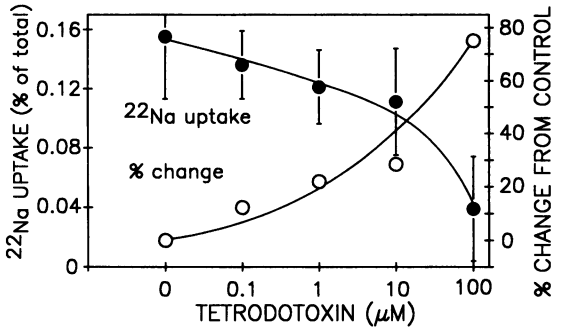

FIG. 2. Na^+ uptake (under counterflux conditions) into proteoliposomes at increasing TTX. Data are presented as radioisotope taken up as a percentage of the total in solution (●) and as percentage change in uptake compared to control (in the absence of TTX) (○).

Table 1. Osmotic volume and TEA (10 mM) effects on K^+ accumulation into liposomes with and without reconstituted protein

	Treatment	K^+ uptake, nmol per mg of lipid	Vol, μl per mg of lipid	Internal K^+, mM
Exp. 1				
P	Control	2.47 ± 0.32	0.21 ± 0.05	11.7 ± 1.6
	+ TEA	1.62 ± 0.38		7.6 ± 1.7
L	Control	1.31 ± 0.26	0.24 ± 0.05	5.5 ± 0.7
	+ TEA	1.25 ± 0.17		5.3 ± 0.7
Exp. 2				
P	Control	25.3 ± 2.2	3.69 ± 0.13	6.9 ± 0.6
	+ TEA	16.5 ± 2.1		4.5 ± 0.6
L	Control	19.7 ± 2.7	5.97 ± 0.04	3.3 ± 0.5
	+ TEA	20.6 ± 1.4		3.4 ± 0.2

Liposomes (L) were prepared in the same manner as proteoliposomes (P), except buffer was loaded onto Sephadex columns instead of detergent-extracted protein. It should be noted that for Exp. 1, sonication times used (two 30-s bursts) were longer than those that were adopted for later experiments; hence, the smaller internal volumes and lower amount of K^+ uptake. In all cases, the reaction solution contained $^{86}Rb^+$ and 10 mM KCl.

Table 2. Channel blocker effects on Na^+ uptake into proteoliposomes

Treatment	Na^+ uptake, nmol per mg of lipid	Inhibition, %
Control	19.6 ± 1.6	—
Amiloride (500 μM)	14.4 ± 1.1	26.5
4-Aminopyridine (100 μM)	17.4 ± 0.9	11.2
TEA (10 mM)	15.3 ± 1.1	21.9
TTX (10 μM)	11.7 ± 1.0	40.3
Lidocaine (100 μM)	15.1 ± 1.4	23.0

All treatments included 10 mM NaCl in the incubation medium. Assay inhibitor concentrations are in parentheses. It should be noted that the concentrations of inhibitors used were all within the range of values previously reported to affect channel blockage. As some of the inhibitors were added from stocks made up in ethanol, all treatments included 0.5% ethanol.

Table 3. Effect of counterion movement on Na^+ uptake into proteoliposomes

External pH	Anion	Na^+ uptake, nmol per mg of lipid
8.0	Cl	21.5 ± 2.1
8.0	Glu	19.5 ± 1.2
7.0	Cl	4.7 ± 1.3
7.0	Glu	5.8 ± 0.9

At the initiation of Na^+ uptake, internal pH of proteoliposomes was 8.0. Uptake of Na^+ was measured in incubation medium that contained $^{22}Na^+$ and 10 mM NaCl (Cl) or sodium gluconate (Glu). Incubation medium (external) pH was adjusted to a final pH of 8.0 or 7.0 with Hepes/Tris.

retaining channel selectivity for alkali over metal cations (10). Also germane is the finding that the K^+- and Na^+-conducting nicotinic acetylcholine receptor is sensitive to amine anesthetics (21). Thus, it can be speculated that cation conductances occurring in proteoliposomes with reconstituted envelope protein as documented here may be facilitated by a "rudimentary" monovalent cation channel sensitive to a range of pharmacologically active monovalent cation channel blockers.

The assertion that Na^+ and K^+ fluxes into proteoliposomes (Fig. 1) are facilitated by a single class of monovalent cation channels was further tested in the series of experiments shown in Fig. 3. A Lineweaver–Burk plot (Fig. 3*A*) identified Na^+ as a competitive inhibitor of K^+ uptake. A Dixon plot identified K^+ as a competitive inhibitor of Na^+ flux (Fig. 3*B*). Although these data are consistent with the aforementioned assertion, an alternative possibility is not ruled out. Perhaps Na^+, entering via a distinct Na^+ channel, reduces the electrochemical gradient driving K^+ uptake (occurring through a separate K^+ channel). This alternative hypothesis was addressed in the experiment shown in Fig. 3*C*. K^+ uptake was measured in the presence of Na^+ or TPP^+, a lipophilic cation. TPP^+, upon diffusing into the proteoliposomes, should mimic the effect of Na^+ on K^+ uptake if the effect of Na^+ is not mediated by competition with K^+ at the outer pore of channels. The Lineweaver–Burk plot of K^+ uptake in the presence of TPP^+ is parallel to the control line, demonstrating uncompetitive uptake (in contrast to the Na^+ effect). These results, along with the work demonstrating cross-sensitivity of K^+ and Na^+ fluxes to channel blockers, are consistent with the hypothesis that the chloroplast envelope-derived K^+-conducting channel may actually be a nonspecific monovalent cation channel. The possibility that Na^+ and K^+ flux into proteoliposomes was facilitated by a Na^+/K^+ cotransporter (23) as opposed to a channel was discounted by further work (data not shown) that demonstrated that flux was insensitive to Na^+, K^+, and Cl^- stoichiometries and was not affected by the inhibitors bumetanide or furosemide.

Some further work was undertaken with intact isolated chloroplasts in order to examine the putative monovalent cation channel in its "native" membrane system. Chloroplasts were isolated and cation uptake was measured as described in ref. 4. In a series of experiments, the channel blocker amiloride was found to restrict K^+ (i.e., $^{86}Rb^+$) or $^{22}Na^+$ uptake into the stroma equally well (data not shown). These results suggest that the protein may also function as a monovalent cation channel in its native configuration in the chloroplast envelope.

Electrochemical Driving Force for Passive Cation Flux into Proteoliposomes. Cation equilibration into the osmotic volume of proteoliposomes should be rate limited by electroneutral exchange across the membranes. In all likelihood, therefore, diffusion gradient-driven cation uptake into proteoliposomes occurs coincident with H^+ efflux and/or anion uptake. In all experiments presented in this report, the

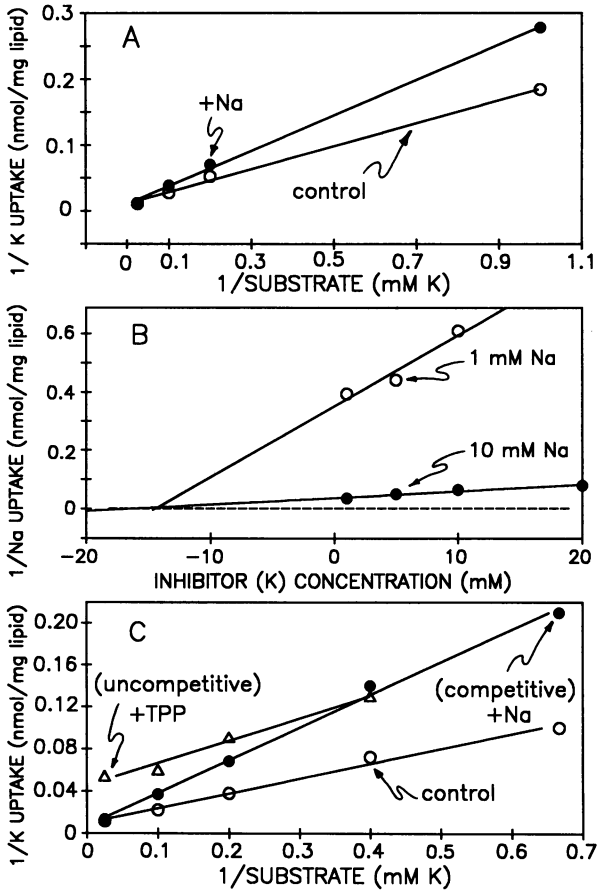

FIG. 3. Competition by Na^+ and K^+ for uptake into proteoliposomes. In one experiment, uptake of K^+ at a range of reaction solution K^+ concentrations was measured in the presence (●) or absence (○) (i.e., control) of 10 mM Na^+ (*A*). In a second experiment, Na^+ uptake was measured at either 1 mM (○) or 10 mM (●) Na^+ and in the presence of various concentrations of K^+ in the reaction solution. In this case, data are presented as a Dixon plot (*B*), where the intersecting lines represent competitive inhibition. In a third experiment (*C*), K^+ uptake at a range of K^+ concentrations was measured in the absence (○) or presence (●) of 10 mM Na^+ or in the presence of 10 mM TPP^+ (△).

monovalent cations have been added as chloride salts. The chloroplast envelope is known to be permeable to Cl^- (6). It could be speculated, therefore, that K^+ (or Na^+) flux through the cation channel (e.g., as shown in Table 1) would be affected by Cl^- fluxes. Results shown in Table 3 suggest otherwise. In this experiment, the effect of replacing Cl^- with an impermeant anion such as gluconate (19) was compared with the effect of altering the proton gradient across the proteoliposome membrane. Replacing Cl^- with gluconate had only a minor, statistically insignificant inhibitory effect on Na^+ uptake into the osmotic volume of proteoliposomes when there was no pH gradient imposed across the membrane and no inhibitory effect at all at an external pH of 7.0 (Table 3). The restriction of H^+ efflux by imposition of a pH gradient ($H_{out} \gg H_{in}$), in contrast, caused a substantial reduction in Na^+ uptake (Table 3). These data suggest that Cl^- uptake is likely not occurring to a great extent and in any case does not influence Na^+ uptake into proteoliposomes as a charge-balancing mechanism. Rather, the bulk of electroneutral exchange upon Na^+ uptake is likely afforded by H^+ efflux from the proteoliposome interior.

Conclusions. On the basis of several lines of evidence presented in this report (inhibitor studies, counterflux assays, and comparison of uptake into proteoliposomes versus liposomes), it can be concluded that (*i*) one of the intrinsic

membrane proteins of the chloroplast inner envelope is a K^+-conducting ion channel, (*ii*) this protein can be detergent-extracted from purified preparations of inner envelope membrane vesicles, and (*iii*) it can be functionally reconstituted into liposomes. The major conclusion of this report—that one intrinsic protein of the chloroplast inner envelope is a monovalent cation channel—is primarily supported by two lines of evidence. The first is that a K^+ channel blocker (TEA) was found to reduce cation uptake in liposomes only in the presence of reconstituted chloroplast envelope protein. It should be noted that cation uptake into proteoliposomes was found to be sensitive to TEA in a total of four other experiments (data not shown). A second line of evidence involves the demonstration of radioisotope movement under counterflux conditions. In addition to the work shown in Fig. 1, K^+ and/or Na^+ uptake was demonstrated to occur under counterflux conditions in six other experiments (data not shown). Additional work under counterflux conditions further substantiated the contention that the rapid accumulation and then loss of radiolabeled cation into proteoliposomes as shown in Fig. 1 was due to the presence of a channel protein. At a point of rapid radioisotope uptake under counterflux conditions, depolarization of the membrane (by addition of external K^+ or Na^+) should lead to immediate label efflux, if label uptake occurs through a channel (12). Exposure of proteoliposomes to such depolarizing conditions at a point of maximum label uptake was found to result in instantaneous loss of internalized label (data not shown). Conversely, addition of a channel blocker to proteoliposomes at a point of maximum uptake should reduce the rate of label efflux during the latter portion of the counterflux time course. Both amiloride and TEA were tested under these conditions and found to reduce the rate of label efflux (data not shown). These results further support the contention that the cation fluxes shown to occur across the proteoliposome membranes (e.g., see Fig. 1) were due to the presence of a functional chloroplast envelope channel protein.

This work provides an initial characterization of one component (i.e., the K^+ uniport pathway) of the system facilitating H^+/K^+ counterfluxes across the envelope; these counterfluxes have a profound effect on stromal pH and regulation of photosynthesis (1, 2, 5, 6). Relatively little is known about ion channels in organelle membranes of plant cells; this report contributes to this knowledge base. Further studies of the molecular nature of this transport protein may lead to the elucidation of intriguing differences between this ion channel and similar channels characterized in animal systems. Finally, the demonstration here of functional reconstitution offers a potential method by which the channel could be purified away from other proteins in detergent extracts of chloroplast inner envelope membranes.

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1. Werdan, K., Heldt, H. W. & Milovancev, M. (1975) *Biochim. Biophys. Acta* **396**, 276–292.
2. Wu, W. H. & Berkowitz, G. A. (1992) *Plant Physiol.* **98**, 666–672.
3. Peters, J. S. & Berkowitz, G. A. (1991) *Plant Physiol.* **95**, 1229–1236.
4. Wu, W. H. & Berkowitz, G. A. (1991) *Plant Physiol.* **97**, 1551–1557.
5. Maury, W. J., Huber, S. C. & Moreland, D. E. (1981) *Plant Physiol.* **68**, 1257–1263.
6. Demmig, B. & Gimmler, H. (1983) *Plant Physiol.* **73**, 169–174.
7. Strong, P. N. (1990) in *Potassium Channels*, ed. Cook, N. S. (Halsted, New York), pp. 23–39.
8. Wu, W. H. & Berkowitz, G. A. (1992) *Plant Physiol.* **99**, 553–560.
9. Kawaga, Y. & Racker, E. (1971) *J. Biol. Chem.* **246**, 5477–5487.
10. Stanfield, P. R. (1983) *Rev. Physiol. Biochem. Pharmacol.* **97**, 1–67.
11. Howitz, K. T. & McCarty, R. E. (1991) *Plant Physiol.* **96**, 1060–1069.
12. Garty, H., Bernardo, R. & Karlish, S. J. D. (1983) *J. Biol. Chem.* **258**, 13094–13099.
13. Albuquerque, E. X., Daly, J. W. & Warnick, J. E. (1988) in *Ion Channels*, ed. Narahashi, T. (Plenum, New York), Vol. 1, pp. 95–150.
14. Gellens, M. E., George, A. L., Jr., Chen, L., Chahine, M., Horn, R., Barchi, R. L. & Kallen, R. G. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 554–558.
15. Kleyman, T. R. & Cragoe, E., Jr. (1988) *J. Membr. Biol.* **105**, 1–21.
16. Stoelting, R. K. (1987) *Pharmacology and Physiology in Anesthetic Practice* (Lippincott, Philadelphia), p. 327.
17. Yeh, J. Z., Oxford, G. S., Wu, C. H. & Narahashi, T. (1976) *J. Gen. Physiol.* **68**, 519–535.
18. Swandulla, D. & Partridge, L. D. (1990) in *Potassium Channels*, ed. Cook, N. S. (Halsted, New York), pp. 167–180.
19. McKinley, D. & Meissner, G. (1978) *J. Membr. Biol.* **44**, 159–186.
20. Wilcox, C. S. (1987) *Curr. Top. Membr. Transp.* **28**, 331–350.
21. Dawson, D. (1987) *Curr. Top. Membr. Transp.* **28**, 41–71.
22. Akaike, N. & Takahaski, K. (1992) *J. Physiol.* **450**, 529–546.
23. Lauf, P. K., McManus, T. J., Haas, M., Forbush, B., III, Duhm, J., Flatman, P. W., Saier, M. H., Jr., & Russell, J. M. (1987) *Am. Soc. Exp. Biol. Fed. Proc.* **6**, 2377–2394.