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Dissipation of the Proton Electrochemical Potential in Intact and Lysed Chloroplasts[1]

I. The Electrical Potential

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ABSTRACT

Effective ionophore:chlorophyll ratios were determined for various ionophores that decrease the electrical potential across thylakoid membranes in intact and hypo-osmotically lysed chloroplasts isolated from spinach (*Spinacia oleracea*). The efficacy of gramicidin D, valinomycin, carbonylcyanide *m*-chlorophenylhydrazone, and dicyclohexano-18-crown-6 in collapsing the electrical potential was determined spectrophotometrically by the decay half-time of the absorbance change at 518 nanometers induced by a saturating, single turnover flash. The results show that the effectiveness of the ionophores in collapsing the electrical potential in intact and lysed chloroplasts depends on the amount of ionophore-accessible membrane in the assay medium. Only gramicidin exhibited a significant difference in efficacy between intact and lysed chloroplasts. The ratio of gramicidin to chlorophyll required to collapse the electrical potential was more than 50 times higher in intact chloroplasts than in lysed chloroplasts. The efficacy of carbonylcyanide *m*-chlorophenylhydrazone was significantly reduced in the presence of bovine serum albumin. The other ionophores tested maintained their potency in the presence of bovine serum albumin. Valinomycin was the most effective ionophore tested for collapsing the electrical potential in intact chloroplasts, whereas gramicidin was the most potent ionophore in lysed chloroplasts. The significance of the ionophore:chlorophyll ratios required to collapse the electrical potential is discussed with regard to bioenergetic studies, especially those that examine the contribution of the transmembrane electrochemical potential to protein transport into chloroplasts.

The proton electrochemical potential, a combination of $\Delta\Psi$[3] and the proton chemical potential (proportional to ΔpH) across the membrane, provides the energy required for ATP synthesis (19) and other membrane transport processes, including protein import in mitochondria (23, 31). The relationship between membrane energization and transport processes has been studied extensively using compounds that dissipate the proton electrochemical potential. Among these compounds are ionophores, which are small hydrophobic molecules that mediate the transport of ions across lipid barriers. Their modes of action are reasonably well understood (14, 19, 25), although the specific mechanism is a topic of continued research (*e.g.* 24, 26). In the work described here, four ionophores, valinomycin, CROWN, CCCP, and gramicidin, were used to collapse $\Delta\Psi$ across thylakoid membranes. Valinomycin, a naturally occurring antibiotic, and CROWN, a synthetic molecule, are classified as neutral, mobile ionophores. They act as electrical uniporters that transport a single cation (in this case K^+) down an $\Delta\Psi$ gradient. Valinomycin and CROWN are particularly useful because they collapse $\Delta\Psi$ without dissipating the pH gradient. CCCP, a lypophyllic, weak acid that is soluble in the lipid domain of the membrane in both the protonated and deprotonated form, is an electrogenic proton carrier (a proton uniporter). The protonated form carries protons down the proton electrochemical gradient, whereas the negatively charged deprotonated form is driven to the positive side of the membrane by the electric field. The result is a net transfer of protons across the membrane and a collapse of ΔpH and $\Delta\Psi$. Gramicidin, another naturally occurring antibiotic, forms a channel in the lipid bilayer that allows alkali cations and protons to diffuse across the membrane. Compounds like gramicidin and CCCP, which collapse both components of the proton electrochemical gradient, are effective uncouplers of ATP synthesis in thylakoid vesicles (7). In contrast, ionophores that collapse $\Delta\Psi$ but not ΔpH (*e.g.* valinomycin and nonactin) have little effect on ATP synthesis in steady-state light, but are effective uncouplers for a short period at the onset of illumination (21).

The ability of ionophores to collapse the electrical, chemical, or both components of the electrochemical proton potential makes them useful agents to investigate bioenergetic mechanisms in thylakoid vesicles and intact chloroplasts. However, because few instruments for determining the size of $\Delta\Psi$ exist, and because determining ΔpH in intact chloroplasts is difficult, several researchers have had to use ionophores and uncouplers without demonstrating their ability to

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[3] Abbreviations: $\Delta\Psi$, electrical potential; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CROWN, dicyclohexano-18-crown-6; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; ΔpH, pH difference across the membrane.

completely collapse the electrochemical potential (5, 22, 27, 30). Although effective concentrations of some chloroplast uncouplers and ionophores have been compiled for thylakoids, data for intact chloroplasts are scant, and caution is advised in considering such compilations because of pH effects and differential partitioning of the compounds in chloroplast membranes (*e.g.* 1, 6, 7, 15, 28). As a consequence, the dissipation of $\Delta\Psi$ and/or ΔpH in intact chloroplasts has often been inferred from results obtained using thylakoid vesicles. The work described here addresses this problem by assessing the efficacy of various ionophores in collapsing $\Delta\Psi$ across thylakoid membranes in intact and lysed chloroplasts. We determined: (a) the dependence of the effectiveness of valinomycin, CROWN, CCCP, and gramicidin on the membrane concentration; (b) the impact of BSA in the assay medium on the potency of the ionophores; (c) the significance of the order of addition of ionophore and membrane sample; and (d) the time required for apparent equilibration of the ionophores in the membrane-containing assay medium. The relative rate of ion movement across thylakoid membranes was determined by the decay kinetics of the electrochromic shift, monitored by the flash-induced absorbance change at 518 nm. The objective is to provide a guide for using ionophores to collapse $\Delta\Psi$ in intact chloroplasts under conditions commonly used to study chloroplast energetics and transport phenomena. The effectiveness of various uncouplers in collapsing ΔpH will be presented in a subsequent report.

MATERIALS AND METHODS

Plant Growth Condition

Hydroponic

Spinacia oleracea plants (U.S. 424; Ferry-Morse Seed Co., Mountain View, CA)[4] were hydroponically grown using a modified Hoagland nutrient solution (11). The concentration of the listed components was changed to the following concentrations: 9 mM KNO_3, 4 mM $Ca(NO_3)_2$, and 1.4 μM $NaMoO_4 \cdot H_2O$. Additionally, 0.5 mM $MgCl_2$ was included. Plants were grown in controlled-environment growth chambers—12-h light period with a constant irradiation of about 300 μE m^{-2} s^{-1}, 20°C constant temperature, and a RH between 60 and 70%.

Soil

Spinach plants were also soil-grown in controlled-environment growth chambers—12-h light period with a constant irradiation of about 450 μE m^{-2} s^{-1}, 20°C light temperature, 18°C dark temperature, and a RH of about 75%. The plants were grown in pots of a 2:1:1 mixture of soil, peat, and perlite, respectively; fertilized twice per week with Miracle Gro (Stern's Nursery, Inc., Geneva, NY); and watered daily with deionized water. Treatment response of chloroplasts isolated from soil or hydroponically grown plants was the same.

[4] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or the University of Illinois and does not imply its approval to the exclusion of other products or vendors that may be suitable.

Chloroplast and Thylakoid Preparation

Intact chloroplasts were isolated from leaves harvested within 2 h of the start of the photoperiod or from leaves stored up to 2 weeks in the dark at 5°C with petioles in deionized H_2O. Intact chloroplasts were isolated essentially as described by Jensen and Bassham (12). All procedures were done at 0 to 4°C unless noted otherwise. Three to five leaves were deveined; cut with a single-edged razor blade into pieces about 1 cm^2; placed in 100 mL buffer composed of 330 mM sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, and 0.1% (w/w) defatted BSA (no isoascorbate), pH adjusted to 6.5 at 2°C with HCl; and homogenized by two or three short bursts of a Brinkman Homogenizer, model PT 10/35. The homogenate was filtered through four layers of miracloth and immediately spun in a Beckman swinging bucket rotor JS-13 at 1200*g* for 1 min. The pellets were gently resuspended, using a small camel hair paintbrush, in approximately 4 mL resuspension buffer composed of 50 mM Hepes, 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, and 0.2% (w/w) defatted BSA, pH adjusted to 7.6 at 2°C with KOH; overlaid on a 4-mL solution of resuspension buffer containing 40% Percoll; and centrifuged for 1.5 min at 1200*g* in the swinging bucket rotor. The pelleted intact chloroplasts were again resuspended with a small camel hair brush in about 0.5 mL resuspension medium to keep the final Chl concentration between 2 and 4 mM.

Thylakoids were prepared in a 4-mL plastic cuvette by hypo-osmotically shocking intact chloroplasts in 1.1× resuspension buffer (described above) without sorbitol. The osmotic shock was carried out in cuvettes on ice, at 18°C, or at room temperature just after the buffer had been removed from the ice bucket. After 1 to 2 min, sorbitol was added, which resulted in a 1× resuspension buffer as described above, except in some cases 5 mM $MgCl_2$ was included. The different treatments did not affect the results. After the final component was added, the cuvette was placed in a thermostatically controlled water-jacketed cuvette holder for at least 2 min before measurements were started.

Chl

Chl concentration in 80% (v/v) acetone:water was determined spectrophotometrically at 647 and 664 nm (−730 nm) using the extinction coefficients of Ziegler and Egle (33).

Chloroplast Intactness

Chloroplast intactness (routinely 85–95%) was determined by phase contrast microscopy (29).

The Electrochromic Shift at 518 nm (ΔA518)

Absorbance changes at 518 nm (4 nm half-bandwidth) were monitored after single turnover flashes (xenon flash lamp, FX-193; EG & G Electro Optics, Salem, MA) filtered by a red blocking filter, Corning CS 2-58 (Corning Glass Works, Corning, NY) using a laboratory-built spectrophotometer described previously (2). The measuring beam pathlength was 1 cm. In one set of experiments, the absorbance pathlength was decreased to 1 mm because the Chl concentration was varied

from 160 to 650 μM. The chloroplast suspensions were maintained at 18°C during measurements by a thermostatically controlled cuvette-jacket.

Chloroplast Stability

At 18°C, the extent of the ΔA518 due to a single turnover flash is more stable in intact chloroplasts than in lysed chloroplasts. However, the decay kinetics of the ΔA518 in intact and osmotically lysed chloroplasts were essentially identical over a 10 to 20 min period. Sampling was completed in less than 20 min.

Ionophore Addition

Gramicidin D (a mol wt of 1800 was used) and valinomycin were purchased from Sigma Chemical Co. (St. Louis, MO). CROWN was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), and CCCP was from E. I. Dupont De Nemours & Co. (Wilmington, DE). Ethanolic stock solutions of all ionophores were utilized. The addition of ethanol equivalent to a final concentration of 10% (v/v) did not significantly affect the kinetics of the absorbance measurements during the first 40 ms following a flash (data not shown). For the results shown, the final concentration of ethanol at the highest ionophore concentration used never exceeded 10% v/v (CROWN). Preparations with the highest gramicidin and valinomycin concentrations used contained 8.5% ethanol, and the highest CCCP preparations contained 2.1%. In Table I, the highest ethanol concentration used was 10% for CROWN and 4.3% in the caes of 13 μM gramicidin and intact chloroplasts. However, high concentrations of ethanol are not recommended. An effective valinomycin concentration of 0.9 μM in a 30 μM Chl solution contained 0.3% ethanol.

Since gramicidin is extremely hydrophobic and difficult to wash from membranes (1), the order in which the chloroplasts and ionophores are added may affect the efficacy of gramicidin at low concentrations. Indeed at low gramicidin concentrations (for example, 0.01 μM gramicidin and 16 μM Chl, which is a 6.3×10^{-4} gramicidin:Chl ratio), gramicidin was more effective when added before the chloroplasts (data not shown). At higher concentrations, the order of addition did not significantly affect the results. The order of addition of the other ionophores did not matter (28). For the data presented here, ionophores were added to the resuspension buffer in cuvettes before addition of intact chloroplasts. Ionophores were the last component added to osmotically lysed chloroplast samples.

It is also noteworthy that the concentration of the stock gramicidin appeared to alter the efficacy of gramicidin (data not shown). Gramicidin from a higher concentration stock solution was less effective than from a lower concentration stock solution. The extreme hydrophobicity of gramicidin may explain these findings. That is, more gramicidin may come out of solution from higher stock concentrations when added to the buffer. Gramicidin was added from a stock solution of 300 μM because it allowed the greatest range of final concentrations without a high final ethanol concentration.

The decay of the ΔA518 varied during the first few minutes after the addition of gramicidin, but was reasonably stable after about 5 min (data not shown), so gramicidin data were collected at least 5 min after addition of the final component. The effect of the other ionophores was stable within 2 min, and measurements for these ionophores were begun at least 2 min after preparation of the complete reaction mixture.

RESULTS

ΔA518—ΔΨ across the Thylakoid Membrane

In most photosynthetic organisms, an electric field created across the thylakoid membrane induces an electrochromic shift in the carotenoid and Chl b antenna molecules that causes an absorbance increase with a peak near 518 nm (reviewed in ref. 32). The absorbance increase induced by a short flash (ΔA518) is due to the rapid, initial charge separation within PSII and PSI (2, 32) and a slower absorbance change that has been attributed to electrogenic charge transfer steps within the cytochrome bf complex (13) (Fig. 1A, con-

Table I. *Effective ionophore: Chl ratios for collapsing the ΔΨ (ΔA518) in intact and lysed chloroplasts*

The ratios shown are the ionophore: Chl ratio required to decrease the decay half-time of ΔA518 to less than 200 μs. When present, BSA was 200 μg/ml. Also shown are concentrations of ionophores required to collapse ΔΨ in a sample at a Chl concentration of 30 μM. The data are taken from Figure 2. Further details are given in the text.

Ionophore	Experimental Material	Ionophore: Chl Ratio	Example of [Ionophore] for [Chl] = 30 μM
Gramicidin D[a]	Intact chloroplasts	0.43	13 μM
	Lysed chloroplasts	0.007	0.2 μM
Valinomycin[a]	Intact chloroplasts	0.03	0.9 μM
	Lysed chloroplasts	0.03	0.9 μM
Crown[a]	Intact chloroplasts	170	5.1 mM
	Lysed chloroplasts	170	5.1 mM
CCCP	Intact or lysed chloroplasts		
	−BSA	5	150 μM
	+BSA	10	300 μM

[a] The results were the same in the presence or absence of BSA.

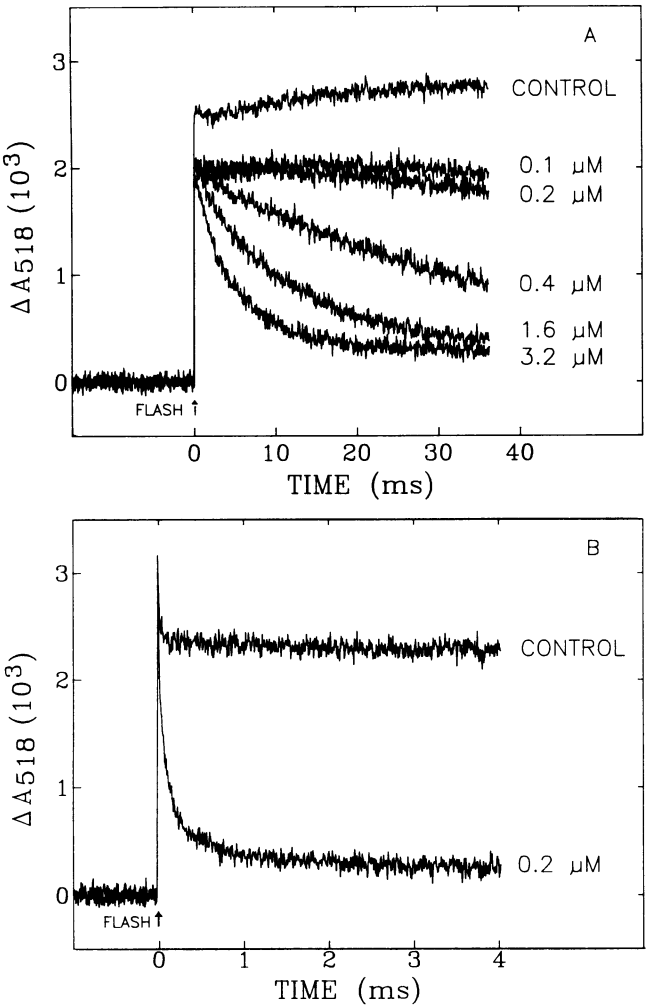

Figure 1. Effects of gramicidin on the electrochromic shift monitored at 518 nm. A, Intact chloroplasts; B, osmotically lysed chloroplasts. Measurements were made as described in "Materials and Methods." Samples contained 30 μM Chl in resuspension buffer with 0.1 mM methyl viologen. Note difference in time scale between A and B. Data in A were collected at an amplifier bandwidth of 20 kHz, and traces represent the average of eight flashes. Data were collected at an amplifier bandwidth of 200 kHz in B and traces represent the average of 32 flashes—two intact samples and two lysed samples, eight flashes each. Flash rate was 0.1 Hz in both cases, and slit width was 4 nm. Control traces from intact and lysed samples were identical.

trol). The decay kinetics of the ΔA518 after a short flash reveal the decay of ΔΨ due to ion movement across the membrane (18, 28, 32), including proton efflux through the ATPase (18). Increasing the membrane permeability to cations and protons with ionophores accelerates the decay of the electric field.

We determined the effective ionophore:Chl ratios for collapsing ΔΨ in spinach chloroplasts and lysed chloroplasts by monitoring the decay kinetics of the ΔA518 following a saturating, single turnover light flash (Table I). Based on kinetic studies of phosphorylation (8, 21), we assume that, if the ΔΨ across the membrane decays to one-half of its maximum value in less than 200 μs, its contribution to energetic processes is negligible. A decay half-time of 200 μs indicates that the dissipation of ΔΨ is at least 5-fold faster than its buildup by the steady-state turnover rate of the reaction centers (17), so ΔΨ is effectively collapsed in continuous illumination.

Gramicidin

Gramicidin was the only ionophore tested that exhibited a distinct difference between intact and hypo-osmotically lysed chloroplasts. Approximately 60 times more gramicidin was required to collapse ΔΨ across thylakoid membranes in intact chloroplasts than in lysed chloroplasts (Figs. 1 and 2A, Table I). Experiments using chloroplasts in the presence and absence of BSA showed that there was no significant interaction of gramicidin with BSA.

Figure 1 shows that 0.2 μM gramicidin:30 μM Chl (gramicidin:Chl = 7×10^{-3}) was sufficient to decrease the decay half-time of the ΔA518 to less than 200 μs in lysed chloroplasts, but had little effect on the decay kinetics in the assay medium containing the intact chloroplasts. However, 0.2 μM gramicidin decreased the extent of the ΔA518 by 15%. These results indicate that gramicidin collapses ΔΨ in the exposed thylakoids before it affects thylakoids bounded by an intact chloroplast envelope. This interpretation is supported by a set of experiments in which six different chloroplast samples were compared for intactness and maximum absorbance increase of the fast rise as a percent of control after addition of 0.1 μM gramicidin to chloroplast preparations containing 30 μM Chl (gramicidin:Chl = 3.5×10^{-3}). As determined by phase-contrast microscopy, the samples were 87 ± 3% (SD) intact and the absorbance change in the samples with 0.1 μM gramicidin was 88 ± 2% (SD) of the control absorbance change. These results indicate that monitoring the flash-induced ΔA518 without gramicidin and at a low gramicidin:Chl ratio offers another method for determining chloroplast intactness.

Valinomycin

Valinomycin was the most potent ionophore tested for collapsing ΔΨ in intact chloroplasts (Fig. 2B, Table I). Valinomycin did not exhibit a significant interaction with BSA, and was equally effective in intact and lysed chloroplasts. In a preliminary report (20), we mistakenly stated that there was a difference in the effectiveness of valinomycin in intact and lysed chloroplasts. Herein we retract the statement.

CROWN

CROWN was the least effective ionophore tested (Fig. 2B, Table I). Over 100 times more ionophore than Chl was required to collapse ΔΨ. It did not interact with BSA, and there was no difference between intact and lysed chloroplasts.

CCCP

CCCP was the only ionophore tested that exhibited a significant interaction with BSA (Fig. 2C, Table I). This confirms the observation by Dilley and Schreiber (4) that BSA removes CCCP from thylakoid membranes. There was no difference between intact and lysed chloroplasts.

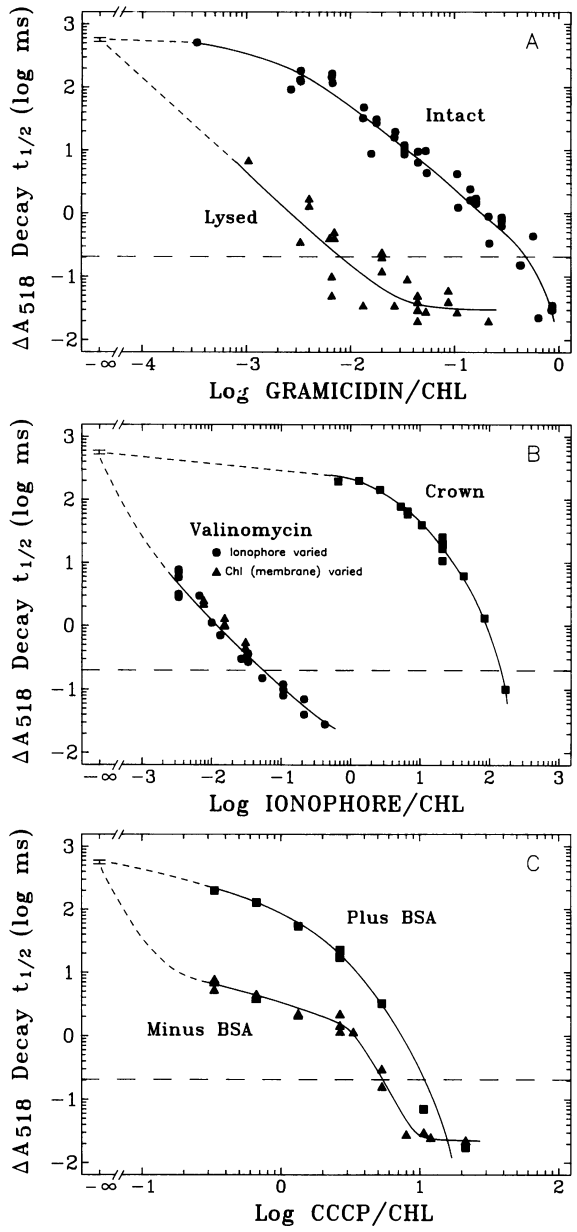

Figure 2. Titrations of the ΔA518 by several ionophores illustrating relationship of ionophore:Chl ratio. A, Gramicidin—lysed (▲); intact (●). All data shown were collected in the presence of 0.2% BSA (w/w). B, Valinomycin treatment in which the ionophore concentration in the assay buffer was varied while the Chl concentration was kept constant (●); valinomycin treatment in which the Chl concentration in the assay medium was varied while the ionophore concentration was kept constant (▲). The Chl concentration was varied from 163 to 650 μM at two constant valinomycin concentrations, 5 and 10 μM. CROWN (■). Includes data with and without BSA and/or additional KCl (KCl was added to test if the buffer contained sufficient K^+ for maximum dissipation of the electrical potential by valinomycin) from intact and lysed chloroplasts. The majority of data included BSA without additional KCl since it was shown that there was little interaction of BSA with valinomycin, and since additional KCl was not required. C, CCCP—plus BSA (■); minus BSA (▲). Includes intact and lysed samples, which exhibited no significant differences. Horizontal dashed lines represent the decay $t_{1/2}$ of the ΔA518 at which it is assumed ΔΨ will make negligible contribution to phosphorylation ($t_{1/2}$ = 200 μs). The symbol (|) represents the ΔA518 $t_{1/2}$ of decay for control samples, 560 ± 40 ms (± SEM, n = 10). Except in B when Chl was varied, absorbance changes were determined in intact or osmotically lysed chloroplasts at a final concentration of 29 to 31 μM Chl (for some experiments, the Chl concentration was as low as 15 μM) in resuspension buffer with 0.1 mM methyl viologen. Each data point represents the average of eight to 32 flashes given at a rate of 0.1 Hz. Data collected at 20 to 200 KHz depending on the degree of uncoupling. Slit width was 4 nm.

DISCUSSION

The experiments described here show that it is the ionophore:Chl ratio rather than the absolute concentration of the ionophore that is important in determining the amount of ionophore required to collapse ΔΨ in chloroplasts and isolated thylakoids. Earlier experiments of a similar nature with gramicidin (15), valinomycin, and nonactin (14, 28) using isolated thylakoids also established a close relationship between the ionophore:Chl ratio.

The significance of these findings is illustrated by work on protein transport into chloroplasts. Several groups have addressed the question of whether the chloroplast envelope membrane needs to be energized to drive import of the small subunit of Rubisco into chloroplasts. The consensus is that ATP is a sufficient energy source, and that a proton electrochemical potential across the chloroplast envelope is not required for import of protein into the stromal phase of chloroplasts (5, 22, 27, 30). The conclusion is based on the demonstration that protein import can be driven by ATP in the presence of ionophores and/or uncouplers that are presumed to collapse the proton electrochemical gradient. Flügge and Hinz (5) used CCCP in combination with valinomycin (no BSA) at concentrations equivalent to a CCCP:Chl and a valinomycin:Chl ratio of 0.01 (calculated assuming a Chl a + b mol wt of 897). Pain and Blobel (22) used CCCP equivalent to a CCCP:Chl ratio of 0.027 and 0.27 in the presence of 0.1 mg/mL BSA. Schindler *et al.* (27) conducted experiments in the presence of 2% BSA and used ionophore concentrations equivalent to a valinomycin:Chl ratio of 0.003, and the highest concentration of CCCP was equivalent to a CCCP:Chl ratio of 0.08. As shown in Table I, the ratios of CCCP:Chl, with or without BSA, used in the above experiments (5, 22, 27) were not sufficient to collapse the ΔΨ across thylakoids of intact chloroplasts. Similarly, the valinomycin:Chl ratio utilized by Schindler *et al.* (27) was insufficient to collapse ΔΨ, whereas that used by Flügge and Hinz (5) decreases the decay halftime to approximately 1 ms (Fig. 2B), which is likely sufficient to collapse the steady-state ΔΨ, although the proton chemical gradient is not dissipated by valinomycin.

In the present study, we did not determine the effectiveness of CCCP and valinomycin in dissipating ΔΨ across the chloroplast envelope. However, since valinomycin and CCCP appear to rapidly equilibrate between membranes (ref. 28 and this work), it is likely that ionophore:Chl ratios that collapse ΔΨ across the thylakoid membrane will also collapse ΔΨ across the chloroplast envelope. In the case in which ΔΨ alone was collapsed, the validity of the interpretation of the data supporting the conclusion that an energized membrane is not

required for protein transport across the chloroplast envelope must be examined. The question of whether the ionophores used were effective in collapsing both components of the proton electrochemical potential remains unanswered.

Grossman *et al.* (9) concluded that import of the small subunit of Rubisco into intact pea chloroplasts does not require a proton electrochemical potential. They showed that the uncouplers salicylanilide XIII or 3,5-dichloro-3-tertbutyl-4′-nitrosalicylanilide inhibited light-mediated uptake of the small subunit, and that the addition of ATP restored the small subunit transport level to the light-mediated level in the presence of 3,5-dichloro-3-tertbutyl-4′-nitrosalicylanilide, but not in the presence of salicylanilide XIII. The import of other polypeptides was not restored to the light-mediated level by ATP, raising the possibility that membrane energization may play a role in import of some proteins.

Recently, Theg *et al.* (30) reinvestigated the bioenergetic requirements of protein import into chloroplasts. In addition to the import of ferredoxin and the small subunit of Rubisco across the chloroplast envelope, they investigated the transport of plastocyanin both across chloroplast envelope membranes and across the thylakoid membrane. Based on experiments using valinomycin, nigericin, and gramicidin, they concluded that protein transport across the chloroplast envelope and the thylakoid membrane is independent of the proton electrochemical potential and requires ATP. In their experiments they used nigericin, an ionophore that mediates the exchange of protons and K^+ across the membrane, thereby collapsing ΔpH without dissipating ΔΨ. The gramicidin:Chl ratio used was 0.015, which is inadequate to collapse ΔΨ (Fig. 2A). The valinomycin:Chl ratio in their experiments was 0.015, which should be adequate to collapse ΔΨ in steady-state light (Fig. 2B). However, since valinomycin was not used in combination with nigericin to collapse ΔpH, protein import was not demonstrated in the absence of an electrochemical gradient. To test the effectiveness of nigericin in chloroplasts, Theg *et al.* (30) showed that nigericin inhibits light-driven import of plastocyanin, presumably by uncoupling ATP synthesis. However, there is a caveat, since in thylakoid vesicles a sizeable threshold proton electrochemical potential is required for ATP synthesis (10), it is possible to uncouple phosphorylation without collapsing the membrane potential.

The energetics of protein import across the chloroplast envelope and thylakoid membranes remains uncertain (16). Cline *et al.* (3) concluded that insertion of the light-harvesting Chl a/b protein into the thylakoid membrane is dependent on a transmembrane electrochemical potential. They showed that in the dark, ATP-driven integration of the protein into the thylakoid membrane was inhibited by uncouplers and ionophores. Insertion was inhibited 70% by valinomycin at a valinomycin:Chl ratio of 0.013.

The difference in the effect of gramicidin on intact and lysed chloroplasts can be explained by the observation that gramicidin is extremely hydrophobic and exchanges slowly between the lipid bilayer and the aqueous phase (1, 28). In our experiments, gramicidin appeared to bind to the chloroplast envelope and did not equilibrate with the thylakoid membranes within 20 min. The differential effect of gramicidin was not related to available lipid, but to the accessibility of gramicidin to thylakoids in the chloroplasts, because the envelope membranes were present in the lysed preparations. The apparent irreversibility of gramicidin partitioning in membranes also accounts for the order of addition effects and time effects discussed in "Materials and Methods." In contrast to gramicidin, our results indicate that valinomycin, CCCP, and CROWN distribute homogeneously among the available envelope and thylakoid membranes. These results can also be explained in terms of the ionophore's hydrophobicity. Ionophores like carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (similar to CCCP) (1) and valinomycin are more readily washed from thylakoids than gramicidin (28), indicating that the ionophores have a greater water solubility than gramicidin.

The data presented here allow us to recommend ionophore:Chl ratios adequate to collapse ΔΨ in intact chloroplasts and isolated thylakoid membranes (Table I). In lysed chloroplast preparations, gramicidin was the most potent ionophore used to collapse ΔΨ across thylakoid membranes. In intact chloroplasts, valinomycin was the most potent ionophore. CCCP was an ineffective ionophore in chloroplasts and thylakoids, and it exhibited a significant interaction with BSA. In addition, CCCP has been shown to inhibit electron transport in thylakoids (7). We are currently developing a technique to monitor the collapse of ΔpH in intact chloroplasts based on the reduction kinetics of cytochrome *f* following exposure to continuous light (manuscript in preparation).

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