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THE EFFECT OF CHLOROPLAST COUPLING FACTOR REMOVAL ON THYLAKOID MEMBRANE ION PERMEABILITY

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SUMMARY

Removal of coupling factor protein (CF_1) from spinach thylakoid membranes results in an enhancement of proton permeability but has no effect on chloride or potassium permeability. Anion permeability was measured by the rate of thylakoid packed volume changes. Potassium permeability was monitored by turbidity changes, packed thylakoid volume changes and ion flux studies using $^{86}Rb^+$ as a tracer. $^{45}Ca^{2+}$ was used to measure divalent cation fluxes. CF_1-depleted chloroplasts had an unaltered rate of Ca^{2+} uptake, but the rate of Ca^{2+} efflux appeared to be increased. Calcium efflux rates could also be increased by the addition of a proton specific uncoupler, FCCP.

INTRODUCTION

One of the current views of energy transduction in chloroplasts holds that proton efflux (inside to outside) through the coupling factor protein $(CF)_1$ provides the driving force for ATP formation [1–3]. That the coupling factor and perhaps the coupling factor binding protein, CF_0, modulate H^+ flux through the membrane is supported by the following observations: (a) Treatment of isolated spinach chloroplasts with EDTA removes the coupling factor protein and increases the proton permeability [4]. (b) Binding of ATP or ADP to the coupling factor decreases the proton efflux rate constant k_d [5]. (c) Certain energy transfer inhibitors such as DIO-9 [6], dicyclohexyl carbodiimide [7], and synthalin [8] interact with CF_1 or CF_0 and decrease the k_d for H^+ efflux. From such observations it is not certain whether the coupling factor acts as a physical block to ion efflux in general by occupying a site that constitutes a hole or port in the membrane, or whether the membrane binding site (CF_0) also contributes to ion selectivity. Indirect experiments [9] using primarily the 520 nm electrochromic shift as an indicator of membrane ionic conductance suggest that removal of CF_1 does not induce significant increased ionic conductance

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; FCCP, carbonyl cyanide-p-trifluoromethoxy-phenylhydrazone; MOPS, morpholino-propane sulfonic acid; Tricine, *N*-tris(hydroxymethyl)-methylglycine.

for ions such as K^+. Another study by Schmid et al. [10] suggests that EDTA extraction of CF_1 leads to an increased ionic conductivity (for ions in addition to H^+) of the membrane, again using the decay of the 520 nm absorbance shift as the criterion for conductivity.

This report more directly tests the hypothesis that removal of coupling factor protein increases ion permeability in general, i.e. exposes an ion port or permeability channel that can accommodate other ions in addition to protons. This test is done by assessing the relative proton permeability of control and CF_1-depleted membranes in comparison to the relative permeabilities of Cl^-, K^+ and Ca^{2+}. We have found that EDTA-treated chloroplasts are not more permeable to K^+ and Cl^- than are control chloroplasts. There are some effects of CF_1 removal on Ca^{2+} efflux, but not influx. This could be due however, to changes in the proton permeability of the membrane due to CF_1 removal. Since there is no clear distinction between actual changes in permeability and changes in ion binding, it is also possible that this faster Ca^{2+} efflux could be due to a proton-limited Ca^{2+}-H^+ exchange.

MATERIALS AND METHODS

Chloroplast preparation and treatments

Chloroplast thylakoid membranes were prepared from spinach leaves as previously described [11], except the resuspension media after the first wash consisted of 0.1 M sorbitol, 1 mM tricine-NaOH (pH 8.0), 0.5 mg/ml bovine serum albumin. EDTA-treated chloroplasts were diluted to about 0.1 mg/ml chlorophyll in 0.1 M sorbitol, 1 mM EDTA and bought to pH 8.0 with NaOH. These were left standing on ice. The control chloroplasts were diluted into the resuspension media mentioned above. After 10 minutes both were centrifuged at $2500 \times g$ for 10 min. For the packed chloroplast volume experiment in Fig. 3 the chloroplasts were washed once in 0.1 M sucrose, 2 mM $MgCl_2$, 10 mM MOPS-NaOH (pH 7.0) and then resuspended in the same. For the light scattering experiment and the packed chloroplast volume experiment in Table II, the chloroplasts were washed once in 0.1 M KCl, 4 mM $MgCl_2$, 10 mM MOPS-NaOH (pH 7.0), 0.5 mg/ml bovine serum albumin and then resuspended in the same. For the ion uptake and efflux studies in Figs. 2 and 4–6, the chloroplasts were washed once in 0.1 M Sorbitol, 2 mM $MgCl_2$, 10 mM MOPS-NaOH (pH 7.0) and resuspended as described in the figure legends.

H^+ flux, phosphorylation and light scattering methods

Proton flux parameters were determined with a Leeds and Northrup microelectrode connected to a Corning model 12 pH meter with the output read on a strip-chart recorder. Photophosphorylation was determined by the pH method [8]. Turbidity changes were monitored on an Aminco model DW-2 spectrophotometer with the cuvette holder set in the position four inches from the photomultiplier tube, so as to increase the amount of scattered light not sensed by the photomultiplier. The optical path length was 1 cm. Packed chloroplast volumes were determined by the microhematocrit method [12]. The free space volume of the pellets was not determined.

Ion fluxes

$^{45}Ca^{2+}$ and $^{86}Rb^+$ uptake and efflux studies were carried out by a technique

similar to that of Gaensslen and McCarty [13]. 0.1 ml of a 10 % sucrose solution was added to a 0.4 ml polyethylene centrifuge tube, then 0.1 ml of a mixture of silicone oils Versilube F-50 and SF-96 (50) in a ratio of 4:1 was layered on top of the sucrose solution. During the experiment, 0.1 ml of a chloroplast suspension was placed on top of these layers and centrifuged for 30 s in a Beckman model 152 microfuge. The tube contents were then frozen in solid CO_2, the bottom phase cut off, and bleached overnight at 65 °C in 1 ml of 30 % H_2O_2 and 3 % Triton X-100. Chlorophyll determinations were carried out by placing the botton phase of the microfuge tube in 0.9 ml of 3 % Triton X-100 and later adding 4 ml of 100 % acetone.

In general, all of the ion uptake and efflux studies were carried out as follows. The control and EDTA treated chloroplast pellets were resuspended in 3 ml of the monovalent or divalent cation solution. These 3 ml contained approximately 10 μCi of tracer in the uptake studies and approximately 100 μCi in the efflux studies. In the uptake experiments, three 0.1 ml aliquots of the chloroplast suspension were taken and centrifuged through silicone oil. For the efflux experiments, after 60 min incubation with the tracer, the chloroplasts were centrifuged at 2500 $\times g$ and then resuspended in 3 ml of media free of the cation being studied. Three centrifuge tubes were loaded just prior to the time indicated. The tubes were kept on ice before and after loading until they were centrifuged.

The results shown in Figs. 2 and Figs. 4–6 are corrected for the amount of label found in the lower phase that comes through the silicone oil with the trapped aqueous volume. The amount of trapped volume that was centrifuged with the chloroplasts was determined by the amount of [^{14}C]inulin found in the lower phase. Internal volume of the chloroplasts was determined by the amount of ^3H_2O found in the lower phase. Analysis of the ^3H_2O content after 1 h in KNO_3 or $Ca(NO_3)_2$ indicated that the internal cation concentration was at least as great as the external concentration. Both the amount of chlorophyll and the trapped volume were found not to vary over the times used in these experiments. Typically approximately 70 μg of chlorophyll were found in the lower phase of each tube, and the trapped volume was 3–5 ‰, i.e. 3–5 % of the amount of [^{14}C]-inulin in the upper phase was found in the lower phase. [^{14}C]inulin was also used to determine the amount of carryover of trapped volume in the chloroplast pellet for the efflux experiments. This is the volume trapped in the pellet during the centrifugation step between loading of the chloroplasts and resuspension in the efflux media. This value was typically 10–30 %. For the efflux experiments, we assumed that the amount of label effluxing into the external media contributed insignificantly to the amount of label in the trapped volume. Therefore, no change was made in the trapped volume correction to account for the slightly elevated isotope concentration in the media.

Radioactivity was measured by liquid scintillation counting on a Searle Isocap/300 using sample channels ratio to monitor quenching. The scintillation fluid used was Tritosol [14].

All of the packed cell volume, proton flux, and light scattering experiments were carried out at 20 °C. The ion tracer studies were carried out at 0 °C.

Valinomycin was purchased from Sigma. ^{45}Ca, ^{86}Rb, ^3H_2O and [^{14}C]-inulin were purchased from Amersham/Searle. The silicone fluids were a gift from Dr. James Frewin, Silicone Products Division, General Electric Corporation, Waterford, N.Y.

RESULTS

Coupling factor removal

In studying H^+ efflux it is necessary to insure that other ion flux processes are not more rate-limiting than H^+. One way this may be done is by adding the K^+ ionophore valinomycin. Table I shows the effect of valinomycin on the rate constant (k_d) of proton efflux through control chloroplast membranes compared to EDTA-treated membranes. EDTA treatment inhibited phosphorylation more than 95 %, and increased the rate constant of proton efflux 2.5-fold. It is not clear if the stimulation of proton efflux rate in the EDTA treated chloroplasts by valinomycin is an effect on all the members of an homogeneous population, or an effect on a smaller group of partially CF_1 depleted (uncoupled) chloroplasts. If we assume an homogeneous population, these results imply that CF_1 removal does not increase K^+ permeability as much as valinomycin does. That is, there is still a limitation on the rate of H^+ efflux in CF_1-resolved particles due to the permeability barrier to potassium inward movement. Valinomycin increases K^+ permeability and permits a more rapid $H_{out}{}^+$-$K_{in}{}^+$ exchange. However, it is not possible to assess from these data whether there is some increase in K^+ permeability due to CF_1 removal. The fact that control membranes showed a significantly greater effect of valinomycin on the H^+ efflux rate constant than CF_1 resolved membranes suggests, but does not clearly establish, that there is an increase in K^+ permeability due to CF_1 removal. (At faster efflux half-times, the response time of the pH electrometer may become limiting).

Turbidity and packed cell volume changes

To provide a clearer test of this possibility we used a technique developed by Avron and coworkers [15, 16] who showed that in the presence of an external permeant anion (such as iodide) and potassium, chloroplasts will swell upon addition of valinomycin. When acetate is the anion, presumably it is only permeant in the protonated form, thus making it necessary to have a "protonophore" present as well as valinomycin in order to initiate swelling. The uncoupler is necessary to carry out

TABLE I

LIGHT-INDUCED EXTERNAL pH CHANGE

Proton pump characteristics as determined by the light-induced external pH change. Chloroplasts (approximately 50 μg/ml chlorophyll concentration) were placed in 100 mM KCl, 4 mM $MgCl_2$, 1 mM MOPS-NaOH (pH 7.0), 10 μM pyocyanin. Rate constants were calculated from the halftime of proton efflux. Phosphorylation rates were 384 μmol ATP/h per mg chlorophyll in the control and no measurable rate in the EDTA-treated chloroplasts.

	Extent $\left(\frac{\mu\text{mol } H^+}{\text{mg chlorophyll}}\right)$	$k_d(s^{-1})$
Control	0.49	0.11
Control +0.5 μM valinomycin	0.48	0.19
EDTA treated	0.05	0.26
EDTA treated +0.5 μM valinomycin	0.05	0.40

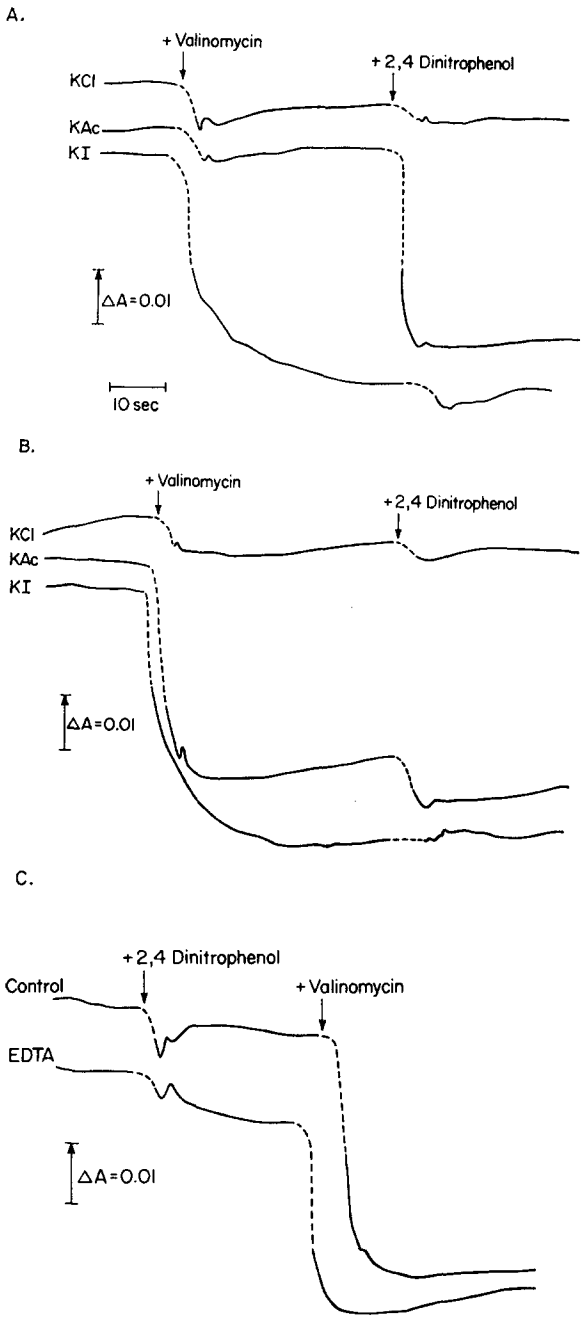

Fig. 1. Turbidity changes at 540 nm. Decreased absorbance indicated swelling. Control and EDTA-treated chloroplasts were 25 and 27 μg chlorophyll/ml, respectively, in solution of 100 mM of the indicated salt, 4 mM $MgCl_2$, 5 mM MOPS-NaOH (pH 7.0). 0.5 μM valinomycin and 50 μM dinitrophenol were added where indicated. A, control chloroplasts in KCl, potassium acetate, and KI; B, EDTA-treated chloroplasts in KCl, potassium acetate, KI; C, control and EDTA-treated chloroplasts in potassium acetate with order of addition of valinomycin and 2,4-dinitrophenol reversed.

TABLE II

PERCENT PACKED CHLOROPLAST (THYLAKOID) VOLUME (PCV) CHANGES

0.2 ml of chloroplast suspension was diluted into 0.6 ml of salt solution as in Fig. 1. This was divided into four aliquots to insure equal chlorophyll content in each of the four different treatments. From each treatment, four "chlorocrit" capillary tubes were prepared. After additions, the chloroplast suspension was spun for 5 min in a microhematocrit centrifuge. Total time from addition of chloroplasts to the salt solution until measurement of percent pack cell volume was 10–11 min. Chlorophyll concentration was normalized to 1 mg/ml. Errors are given as standard deviation. Due to the high chlorophyll content on the stock chloroplast suspensions the separate pipettings for the three salt treatments and for control or EDTA treatments gave a range of percent packed cell volumes, e.g. 9.1, 11.2 and 8.6 percent packed cell volume. Within each of the salt treatments, the PCV values are more reproducible (to approximately 2 %).

Additions	Control			EDTA-treated		
	PCV	Δ PCV	Change (%)	PCV	Δ PCV	Change (%)
KCl						
No addition	9.1±0.2			7.9±0.1		
5 μM valinomycin	9.2±0.1	0.1	1	8.0±0.3	0.1	1
0.5 mM 2,4-dinitrophenol	9.0±0.1	0.1	1	7.9±0.2	0.0	0
0.5 mM 2,4-dinitrophenol +5 μM valinomycin	8.9±0.2	−0.2	2	7.8±0.3	−0.1	1
KI						
No addition	11.2±0.2			9.3±0.6		
5 μM valinomycin	13.5±0.2	1.8	16	11.0±0.1	1.7	18
0.5 mM 2,4-dinitrophenol	10.5±0.1	−0.8	−7	9.1±0.1	−0.2	−2
0.5 mM 2,4-dinitrophenol +5 μM valinomycin	11.5±0.1	0.3	3	10.0±0.1	0.7	8
Potassium acetate						
No addition	8.6±0.0			10.1±0.2		
5 μM valinomycin	8.7±0.2	0.1	1	11.6±0.2	1.5	15
0.5 mM 2,4-dinitrophenol	8.8±0.1	0.2	2	10.2±0.4	0.2	2
0.5 mM 2,4-dinitrophenol +5 μM valinomycin	10.2±0.2	1.5	17	11.8±0.0	1.7	14

the proton that was carried inside with the protonated form of the anion. If the EDTA-treated chloroplasts have enhanced permeability only to protons, valinomycin should be required to initiate swelling in K^+ acetate. Alternatively, if the EDTA-treated chloroplasts also have enhanced potassium permeability, swelling should occur as soon as the chloroplasts are added to the acetate media, and valinomycin or the protonophore would have no effect. Fig. 1 shows that the EDTA-treated chloroplasts are similar to control chloroplasts in that they require valinomycin to initiate swelling in K^+ acetate. The extent of swelling was comparable to that induced by valinomycin in potassium iodide, a case where the anion is permeable with no added compounds. Thus, as expected, the CF_1-depleted membranes are more permeable to protons than the controls. However, there is no significant increase in K^+ permeability in the CF_1-depleted membranes compared to controls, as indicated by the lack of swelling in the K^+ acetate medium.

In order to insure that the observed light scattering changes reflect chloroplast volume changes, independent methods for measuring chloroplast volume were used. Table II shows the percent packed chloroplast (thylakoid) volume changes in the various salt media, induced by valinomycin and 2,4-dinitrophenol. In KCl media there were no significant volume changes in either control or EDTA-resolved membranes, in agreement with the turbidity data of Fig. 1. In KI media, valinomycin induced a 16 and 18 percent increase in packed cell volume in the control and EDTA treatments respectively, as expected since I^- is permeable. For reasons not understood, dinitrophenol caused a significant decrease in the packed thylakoid volume in KI only. Further addition of valinomycin reversed that volume decrease, but the extent of swelling did not reach that seen upon adding just valinomycin. The dinitrophenol-induced shrinkage was not evident in the turbidity changes.

In the potassium acetate media, valinomycin induced a 15 percent increase in the packed volume of the EDTA treated membranes, but no significant change in the control, in agreement with the turbidity data, i.e. the EDTA-treated membranes were leaky to protons but not to K^+.

Monovalent ion movements

Direct quantitative measurements of monovalent cation movements were made using $^{86}Rb^+$ as a monitor of KNO_3 movements, NO_3^- being a permeant anion [16]. The uptake and efflux kinetics are shown in Fig. 2. These data add further evidence that EDTA treatment does not effect either the K^+ influx or the K^+ efflux kinetics in chloroplasts. This was an important additional measurement to make, as it tests whether there may be a difference in K^+ efflux compared to K^+

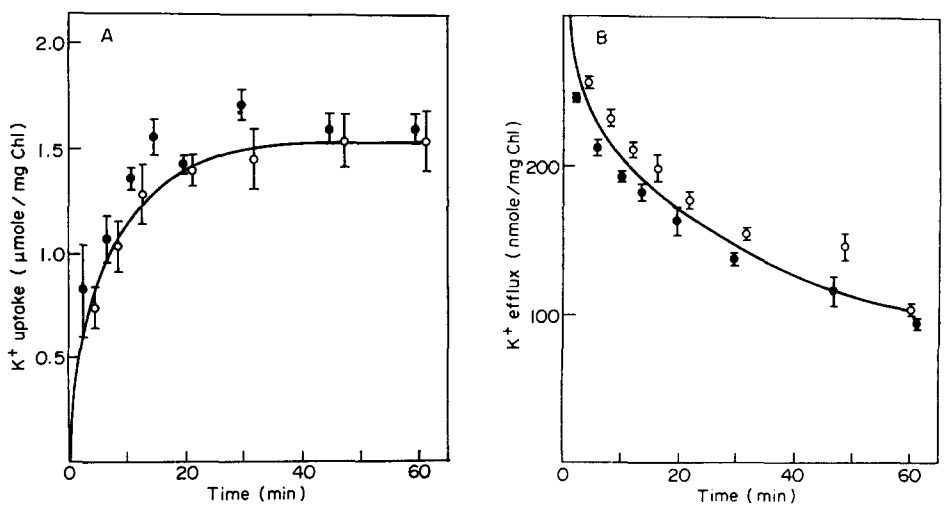

Fig. 2. Uptake and efflux of ^{86}Rb as a measure of KNO_3 movement. A, Chloroplasts were resuspended at $t = 0$ in 0.1 M sorbitol, 30 mM KNO_3, 2 mM $MgCl_2$, 10 mM MOPS-NaOH (pH 7.0), 0.5 mg/ml bovine serum albumin, 5 μM DCMU. B, chloroplasts equilibrated for 1 h in the same media as in A, were centrifuged at 2500 $\times$ g for 10 min and resuspended in media containing 0.1 M Sorbitol, 2 mM $MgCl_2$, 10 mM MOPS-NaOH (pH 7.0), 0.5 mg/ml bovine serum albumin, 5 μM DCMU. ●–●, control; ○–○, EDTA-treated chloroplasts.

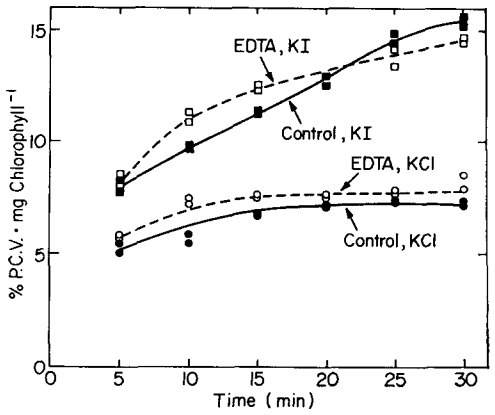

Fig. 3. Percent packed chloroplast (thylakoid) volume (% PCV) changes induced by an increase in external salt concentration. Both control and EDTA-treated chloroplasts were diluted (at $t = 0$) into a medium resulting in a final concentration of 90 mM KCl or KI, 1 0mM sucrose, 2 mM $MgCl_2$, 10 mM MOPS-NaOH (pH 7.0).

influx in control or EDTA-treated membranes. We only measure the efflux kinetics of H^+.

It was also found that 1 μM valinomycin would significantly increase the uptake kinetics of $^{86}Rb^+$ (data not shown). This was taken as evidence that the movements of $^{86}Rb^+$ were limited by a permeability barrier. In addition to this, large packed chloroplast volume changes were observed after similar increases in external K^+ concentration (see Fig. 3). We used these volume changes as an indication that solute was penetrating into an inner osmotic compartment, and that the limiting factor was penetration of a permeability barrier.

It has been reported previously [16] that K^+ movement can be anion limited unless a permeant anion such as I^- or NO_3^- is provided. Fig. 3 shows the anion dependence of swelling induced by an increased external salt concentration, using packed chloroplast volume as the assay. As expected, there is more swelling of control chloroplasts (and CF_1-depleted membranes) in KI compared to KCl. In comparing the rates of swelling in Fig. 3, it is clear that if there had been an increased Cl^- permeability after CF_1 removal, the EDTA-treated chloroplasts would have swelled faster than the control chloroplasts in KCl media. Since this is not the case, it would appear that chloride influx is still a limiting factor for KCl-induced swelling of EDTA-treated chloroplasts.

Divalent ion movements

In order to specifically examine divalent cation permeability, direct measurements of $^{45}Ca^{2+}$ movements were made. Fig. 4 shows that the uptake of Ca^{2+} in control chloroplasts is not anion limited since both $CaCl_2$ and $Ca(NO_3)_2$ have the same uptake kinetics. The uptake of Ca^{2+} has similar kinetics to K^+ uptake ($t_{\frac{1}{2}} = 7$ min for K^+ uptake and 6 min for Ca^{2+}). Fig. 5A shows that Ca^{2+} influx is unaffected by CF_1 removal. We had originally expected the uptake kinetics of $^{45}Ca^{2+}$ to be biphasic with a fast initial phase due to Ca^{2+} binding to external groups on the thyla-

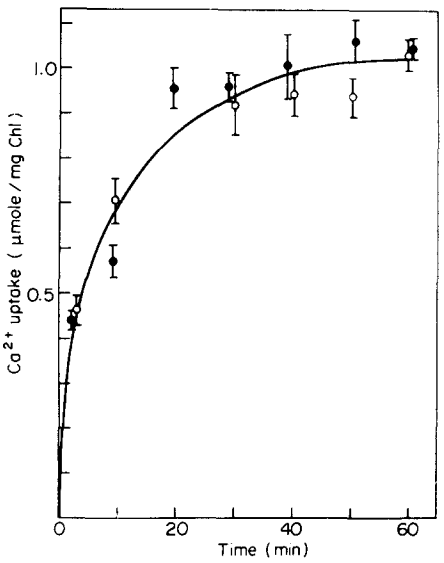

Fig. 4. Uptake of $^{45}Ca^{2+}$ by control chloroplasts in $CaCl_2$ and $Ca(NO_3)_2$. At $t = 0$ a chloroplast pellet was resuspended in 0.1 M Sorbitol, 20 mM $CaCl_2$ or $Ca(NO_3)_2$, 2 mM $MgCl_2$, 10 mM MOPS-NaOH (pH 7.0), 0.5 mg/ml bovine serum albumin, 5 μM DCMU. ●–●, $CaCl_2$; ○–○, $Ca(NO_3)_2$.

koids. However, as Figs. 4 and 5A show, the uptake kinetics appear monophasic. This could be because the amount of fast Ca^{2+} binding is small compared to the total amount of Ca^{2+} uptake. The amount of internal $[^3H]H_2O$ in the thylakoids after a 60 min incubation in $Ca(NO_3)_2$ was sufficient for the internal Ca^{2+} concentration to be 20–30 mM. Assuming that the uptake has gone to equilibrium (as it appears

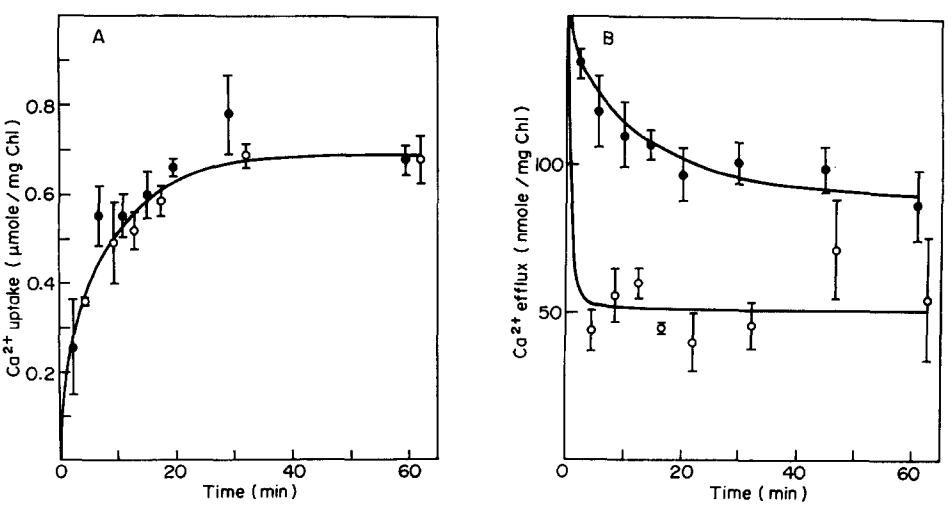

Fig. 5. Uptake and efflux of $^{45}Ca^{2+}$ as a measure of $Ca(NO_3)_2$ movement. Conditions were the same as in Fig. 2 except 20 mM $Ca(NO_3)_2$ were substituted for KNO_3. A, Ca^{2+} uptake. B, Ca^{2+} efflux. ●–●, control; ○–○, EDTA treated chloroplasts.

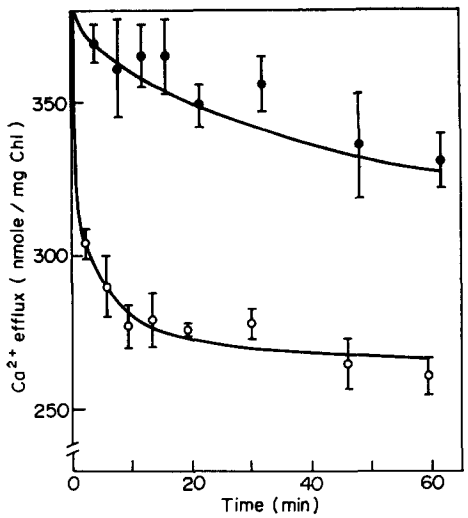

Fig. 6. Uncoupler sensitivity of $Ca(NO_3)_2$ efflux from chloroplasts. Conditions are the same as in Fig. 5 except only control chloroplasts were used. ●–●, control; ○–○, +1 μM FCCP.

to) this indicates that the majority of Ca^{2+} uptake is due to permeation into an inner osmotic compartment.

Fig. 5B shows what appears to be a faster Ca^{2+} efflux from the EDTA-treated chloroplasts, although this efflux apparently is over before we make our first measurement. Either there is a rectification of Ca^{2+} flux in CF_1-depleted membranes, with efflux being faster than influx, or there is some other effect imposed by CF_1 removal. Since more Ca^{2+} is taken up than is necessary for an internal concentration of 20 mM, it is quite possible that this second smaller phase is due to Ca^{2+} debinding, which is either slower than Ca^{2+} efflux, or its movement is limited by different factors than the bulk Ca^{2+} in the internal aqueous phase. Because CF_1 removal greatly increases H^+ conductivity, the increased Ca^{2+} efflux observed in that case may be related to H^+-Ca^{2+} exchange phenomena. If so, then any uncoupler should have the same effect as CF_1 removal. Karlish et al. [15] have shown that 1 μM FCCP can act as a specific proton ionophore. Fig. 6 shows that the efflux of Ca^{2+} from control chloroplasts can also be increased by 1 μM FCCP.

Both potassium and calcium efflux in these experiments apparently have a very fast initial phase. (Compare the extent of uptake in Figs. 3A and 4A to the efflux in Figs. 3B and 4B.) The change in osmolarity used in the beginning of the efflux experiment should cause a chloroplast swelling, but this would not explain the fast efflux observed. Because of this biphasic efflux, we were only able to measure a small fraction of the total ion movement occurring, and changes in this phase only reflect a small amount of the total cation movement.

DISCUSSION

Monovalent ion permeability

The data presented here clearly show that the K^+ and Cl^- permeability of

spinach chloroplast thylakoid membranes is unaltered by EDTA treatment, i.e. removal of coupling factor protein. The CF_1-depleted membranes maintained as much of a barrier to K^+ movement as the control membranes. Experiments measuring thylakoid volume changes by changes in both turbidity and packed cell volume were used as a measure of the permeability barrier to K^+ movement. In potassium acetate-induced swelling, control chloroplasts were shown to have a requirement for both valinomycin and 2,4-dinitrophenol (a proton ionophore) in order to initiate swelling. The CF_1 depleted chloroplasts would swell to the same extent, but only required the addition of valinomycin to initiate swelling. The enhancement of intrinsic thylakoid membrane permeability to protons by CF_1 removal is well documented [4], however it is important to note that valinomycin was still required to initiate swelling in the CF_1-depleted membranes, indicating that a barrier to K^+ influx still existed.

The uptake and efflux kinetics of $^{86}Rb^+$ in the presence of a permeant anion (NO_3^-) were also measured. Both the $^{86}Rb^+$ (K^+) uptake and efflux rates were found to be unaffected by coupling factor removal.

Measurements of the Cl^- permeability of thylakoid membranes were made by following the increase of packed thylakoid volume initiated by an increase in the external potassium salt concentration. In KCl, where Cl^- movement is limiting, and in KI, where K^+ movement is limiting, both control and coupling factor depleted membranes exhibited similar rates of swelling. If chloride permeability had been increased by coupling factor removal, we would expect the thylakoids to swell at a rate limited by K^+ permeability, as is the case in potassium iodide. Since this was not the case it would seem that chloride permeability is also unchanged after CF_1 removal.

Divalent cation permeability

Direct observation of the rate of efflux of $^{45}Ca^{2+}$ from control and EDTA-treated thylakoid membranes indicated that Ca^{2+} efflux was enhanced in the CF_1-depleted membranes (Fig. 5B) in contrast to no effect of CF_1 removal on Ca^{2+} influx (Fig. 5A). However, a similar increase in Ca^{2+} efflux could be observed in control membranes by the addition of a proton-specific ionophore (1 M FCCP). In light of this result it seems probable that Ca^{2+} efflux from control chloroplasts is limited by an H_{in}^+-Ca_{out}^{2+} exchange reaction, perhaps an exchange of Ca^{2+} bound to fixed charges for H^+ binding. Removal of coupling factor protein or addition of an uncoupler removes the proton permeability limitation and this allows a more rapid efflux of Ca^{2+}. In our experiments this efflux rate was too fast to measure accurately, however it seems likely that thylakoid membrane intrinsic Ca^{2+} permeability (judging from the Ca^{2+} uptake experiments) is not changed by the EDTA treatment. This interpretation requires that the Ca^{2+} movements measured here (efflux) are not simply transmembrane passive ion fluxes, but ion binding-debinding phenomena involving membrane fixed charge groups that may be anywhere on the surface or the interior of the membrane. At present this remains speculative.

The possibility exists that the kinetics of ion movement shown may be entirely or in part due to ion binding effects. It seems unlikely that the swelling associated with the increase of external K^+ concentration (see Fig. 3) could be due to any other effect but penetration of solute into an internal osmotic compartment. The fact that valinomycin (see Results) accelerated the uptake of KNO_3 also argues favorably for the uptake being due to permeation. An alternative means of distinguishing permea-

tion from ion exchange phenomena is to measure the internal volume of the thylakoids and the amount of associated ion. If the ion is equilibrating with an inner osmotic space the calculated internal ion concentration will be equal to the external concentration. If however, some ion exchange phenomena occurs in addition to permeation, the calculated internal ion concentration will be greater than the external concentration. The internal thylakoid volume as measured by the uptake of $[^3H]H_2O$ indicated that the internal concentration of Ca^{2+} could be 20–30 mM. This argues against a large portion of the Ca^{2+} uptake measured being bound to the thylakoids. The largest single source of error in the measurement of $[^3H]H_2O$ would be at the bleaching step (see Methods) and this would lead to a loss of $[^3H]H_2O$ and subsequent overestimation of the internal solute concentration. Our finding of a high internal Ca^{2+} concentration could reflect ion binding phenomena, or an overestimation such as this.

Properties of the CF_0 H^+ port

A more fundamental implication of the results presented in this paper concerns the nature of the natural chloroplast protonophore, or CF_0. If the CF_0 port consists of an aqueous channel through which protons can flow, then a smaller ion should also be able to move freely through it. A hydrated proton has a radius of 9 Å as compared to a radius of 3 Å for a hydrated potassium ion, and 6 Å for a hydrated calcium ion [18]. The data presented in this paper indicate that the chloroplast membrane still maintains a barrier to potassium and in some cases calcium movement after removal of the coupling factor protein. This argues strongly against the possibility that the CF_0 hydrophobic protein could be a simple aqueous channel. We are assuming that the CF_0 protein is still at least as good a protonophore after CF_1 removal as it was before removal. If protons traversed the membrane in a hydrated form, then a smaller hydrated monovalent cation should be able to follow the same path. Since this does not occur, then it would seem that if protons appearing on the outside of the membrane after illumination are effluxing from the internal osmotic space, they are crossing the membrane in an unhydrated form. This would be analogous to the case with valinomycin and K^+, where it has been shown [19] that K^+ loses its water of hydration as it enters the valinomycin cage structure. Our results are consistent with the proposal of Williams [20], that the simplest mechanism for proton movement across the membrane is through a region of structured water. Here proton movement should be as fast as in ice, however the movement of other ions would be severely restricted [21].

An alternative mechanism that could provide a selective, passive H^+ transport is a carrier molecule having a fixed charge group(s), such as a carboxyl that would bind H^+ on the inner side and undergo a translational movement to the outside, where the H^+ could debind. Such a mechanism, while possible in principle, seems unlikely for a passive H^+ movement since it might be expected that energy would be required to drive the binding structure back and forth across the lipophilic barrier.

The effect of removal of CF_1 on the decay of the 520 nm field-indicating absorption change was reported by Schmid et al. [9, 10]. They found that EDTA extraction caused the expected increase in decay rate of the 520 nm shift, and it was only partially restored to a lower decay rate by reconstitution with CF_1 whereas the external pH change was completely restored to its original extent and decay rate. They concluded that EDTA extraction leaves the membrane with an increased non-specific conductance that was not decreased by CF_1 reconstitution, a conclusion based solely

on the kinetics of the 520 nm shift. Our data indicates that this increased conductivity is not due to an increase in K^+ or Cl^- permeability, although we cannot rule out the possibility that a divalent cation-proton exchange mechanism could give rise to the observed changes.

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