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The reversibility of freeze/thaw injury to spinach thylakoids; restoration of light-induced proton pumping, membrane-conformational changes and proton gradient formation

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Spinach thylakoids have been frozen under a variety of salt/sucrose concentrations to remove varying amounts of peripheral membrane proteins, including the water-soluble part of the coupling factor complex (CF_1). This leads to a defined degree of uncoupling by exposing the CF_0 proton channel. The ability of thylakoids, subjected to this treatment, to reconstitute light-induced proton pumping, membrane-conformational changes and proton gradient formation when treated with DCCD, an energy transfer inhibitor which interacts with the CF_0 proton channel, thus reducing the proton permeability of the membrane, has been investigated. Full reconstitution of proton pumping and ΔpH formation could be obtained in thylakoids in which up to 75% of the coupling factor complex had been removed by the freezing regime. Even under the most severe conditions employed, in which over 80% of the CF_1 was removed from the membrane, there was still between 25 and 50% reconstitution of proton pumping. Reconstitution of membrane conformational changes as monitored by 90° scattering changes showed a strong positive correlation to the restoration of proton pumping. Reconstitution of slower, light-induced transmittance changes, in contrast, exhibited a more variable response. Little reconstitution of the slow transmittance changes was found under conditions which removed more than 60–70% of the coupling factor complex.

Introduction

On illumination of chloroplasts, the vectorial nature of the electron-transport chain [1] results in the uptake of protons into the intrathylakoid space [2,3]. The resultant proton gradient formed, either free in the lumen as proposed by Mitchell [4], or localised in microenvironments as first proposed by Williams [5], is considered to be the major component of the proton-motive force which results in ATP formation, although the exact coupling mechanism is not yet known [6].

Concomitant with this light-driven proton uptake is a counter exchange of ions (Mg^{2+}) to maintain electroneutrality, and scattering changes which are considered to represent microconformational changes in thylakoid structure [7]. These scattering changes measured in the 90° mode have been convincingly correlated with proton pumping [7–9]. Scattering changes measured in the transmittance mode exhibit a more variable response [10], as there is a strong angular dependency to the

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Abbreviations: DCCD, *N,N'*-dicyclohexyl carbodiimide; CF_1, water-soluble part of the coupling factor complex; Mes, 4-morpholineethanesulfonic acid.

scattering response [10,11], and absorbance changes will also be measured in this mode.

We have recently found that short-term glutaraldehyde treatments of isolated spinach thylakoids selectively abolish the light-induced slow transmittance changes [12], while leaving the 90° scattering changes relatively unaffected. This prompted us to undertake a detailed comparative investigation of these two phenomena [13] in order to provide a more detailed explanation for the transmittance changes.

It has been known for some time that the CF_1 part of the coupling factor complex is bound electrostatically to the membrane [14]. A variety of treatments which can be subdivided into low salt (EDTA [15], pyrophosphate/sucrose [16]), or high salt (NaBr washing [17]), or freezing in the presence of salts [18] have been used to isolate CF_1 and to investigate the nature of the CF_0/CF_1 binding. Dissociation of the CF_1 part of the coupling factor complex from the membrane exposes the CF_0 proton channel and leads to uncoupling of electron transport [16], and dissipation of the light-induced transmembrane electric field [19] by increasing the proton permeability of the membrane [19,20]. It is possible to reconstitute proton pumping by treating the uncoupled membranes with DCCD, an energy transfer inhibitor which blocks the CF_0 channel thus reducing the proton permeability of the membrane [21]. Reconstitution of full photophosphorylation activity by treating the uncoupled membranes with isolated CF_1 has also been extensively investigated [21,22].

We have made use of the knowledge gained by the groups of Heber et al. [23] and Steponkus et al. [24] on freeze/thaw damage to isolated spinach thylakoids to design a range of freezing regimes such that 0–80% of CF_1 was physically removed from the membrane, thus leading to a defined degree of uncoupling. This treatment has the advantage over the other physical treatments in that electron transport, apart from the uncoupling affect, is unaffected. The reconstitution of proton pumping by DCCD treatment of the thylakoids has been compared with the restoration of the light-scattering responses, both in the transmittance and the 90° mode in an attempt to investigate further the physical nature of these two processes.

Materials and Methods

Spinach thylakoids were isolated as in Coughlan and Heber [25].

Freeze / thaw regimes

Thylakoid suspensions (500 μg Chl $\cdot$ ml^{-1}), 100 mM NaCl, up to 250 mM sucrose in 1 ml volume, were placed in a deep freezer (−20°C) for 3 h (cooling rate, $c \cdot 0.5°C \cdot min^{-1}$). Frozen suspensions were thawed at 20°C in a water bath ($c \cdot 2$ min), then stored on ice.

Photosynthetic measurements

Light stimulated phenazine methosulfate (cyclic) or methyl viologen (1,1-dimethyl-4,4′-bipyridinium-dichloride) (linear) -mediated proton uptake and photophosphorylation were measured by medium alkalisation as described by Dilley [26]. Electron transport activities were measured as oxygen consumption/evolution in a Walker-type oxygen electrode [27]. Light-dependent 9-amino acridine fluorescence quenching and scattering changes (90° and 180°) were measured as previously described [13]. SDS-polyacrylamide gel electrophoresis was carried out as described by Piccioni et al. [28]. Gels were fixed and stained as in Ref. 28. Trypsin-activated Ca^{2+} ATPase activity was assayed as in experiments by Lien and Racker [29]. Phosphate determinations were carried out as described by Ames [30]. Chlorophyll determinations in 80% (v/v) acetone extracts were determined according to Arnon [31]. Protein content of supernatant samples which had been clarified by centrifugation (12000 $\times g$ for 10 min at 4°C) was determined by the method of Bradford [32].

Results

When thylakoids were frozen under the employed conditions, there was a progressive uncoupling of linear electon transport at the lower sucrose concentrations (Fig. 1A). This correlated to a loss of photophosphorylation, nine amino acridine fluorescence quenching, and a substantial loss of proton pumping activity (Fig. 1A–C). The increase in ionic permeability brought about by this range of freeze/thaw regimes appeared to be specific for protons. No increase in either the K^+

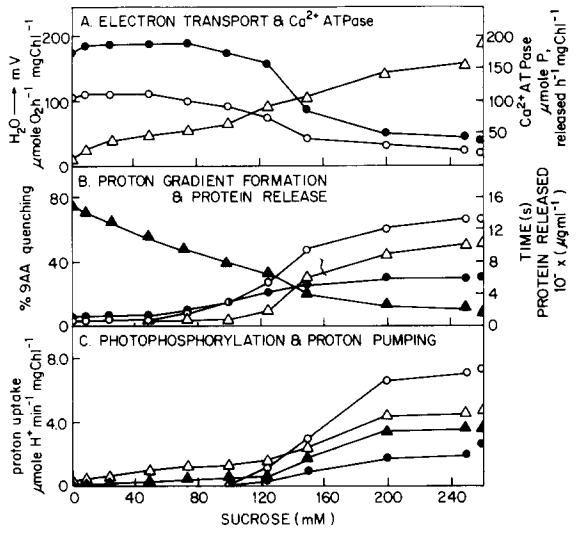

Fig. 1. The effect of freeze/thaw regimes (100 mM NaCl, up to 250 mM sucrose), 3 h, −20°C on (A) linear electron transport ($H_2O \rightarrow$ methyl viologen), (B) light-dependent 9-amino acidine fluorescent quenching and protein release, and (C) photophosphorylation and proton pumping. Experimental conditions: (A) Electron transport (pH 6.7, 7.6) and Ca^{2+} ATPase. The medium (1 ml) contained 100 mM sorbitol/10 mM $MgCl_2$/10 mM tricine-KOH (pH 7.6) or 10 mM Mes/KOH (pH 6.7), 0.1 mM methyl viologen, 50 µg Chl, 20°C. The actinic light (610–800 nm) intensity was 200 $W \cdot m^{-2}$. Ca^{2+} ATPase activities of membrane aliquots was assayed as in Ref. 30. ●———●, pH 7.6; ○———○, pH 6.7; △———△, trypsin-activated Ca^{2+} ATPase, (B) proton gradient formation and protein released into the supernatant. Conditions as in (A) but with the addition of 1 µM 9-amino acridine, final volume, 1.5 ml, 22.5 µg Chl (pH 7.6), excitation wavelength, 400 nm (1 nm slit width); emission wavelength, 480 nm (20 nm slit width). ○———○, percentage 9-amino acridine fluorescence quenching; ●———●, half-time 9-amino acridine fluorescence rise (s); △———△, half-time dark decay (s); ▲———▲, protein present in the supernatant ($\mu g \cdot ml^{-1}$). (C) Photophosphorylation and proton pumping. For photophosphorylation measurements the medium (1 ml) contained 100 mM sorbitol, 10 mM $MgCl_2$, 1 mM tricine-KOH, 1.0 mM KH_2PO_4, 1 mM ADP, and either 0.1 mM methyl viologen or 15 µM phenazine methosulfate, 50 µg Chl, 20°C, actinic light (610–800 nm) intensity was 600 $W \cdot m^{-2}$. For proton-pumping measurements the medium (1 ml) contained 100 mM sorbitol, 10 mM $MgCl_2$, and either 1 mM Mes/KOH (pH 6.7) or 1 mM tricine/KOH (pH 7.6), 0.1 mM methyl viologen, 50 µg Chl, 20°C, actinic light (610–800 nm) intensity, 200 $W \cdot m^{-2}$. All measurements are the mean ± S.D. ($n = 5$). ○———○, Cyclic photophosphorylation (15 µM ▲———▲, proton pumping pH 7.6; ●———●, linear photophosphorylation (0.1 mM methyl viologen); △———△, proton pumping pH 6.7.

or Cl^- permeability of the membrane was observed under these experimental conditions (Coughlan and Demmig, unpublished observations). Concomitant with the uncoupling of electron transport was an increasing release of protein from the membrane into the supernatant (Fig. 1B). SDS polyacrylamide gel electrophoresis of both thylakoid and supernatant fractions show a progressive loss of the α and β subunits of the CF_1 part of the coupling factor complex from the membrane with decreasing sucrose concentrations (Fig. 2). This was correlated with the appearance of the five subunits (α–ϵ) of CF_1 in the supernatant. Under the severest conditions employed (lanes 10–13) 75–80% of the CF_1 was removed from the membrane as shown by immunoblot analysis using antisera raised against the α and β subunits of CF_1 (data not shown). By densitometric analysis of the gels, between 80 and 90% of the total protein released by the differing freeze/thaw treatments was CF_1. Other minor bands visible included both the large (54 kDa) and small (12 kDa) subunits of RuBP carboxylase and peptides of apparent molecular weight 92 kDa, 47 kDa, 32 kDa, 19 kDa and 10 kDa. Most of these peptides were present in constant amounts throughout the whole range of conditions (lanes 1–13). Apart from the CF_1, subunits, peptides which were released from the membrane by increasing amounts of freeze/thaw damage were a 32 kDa peptide, a 19 kDa and 10 kDa peptide which was shown to be immulogically equivalent to plastocyanin by western blot analysis (data not shown). Membrane-bound trypsin-activated Ca^{2+} ATPase activity, a direct enzymatic assay for CF_1, was also progressively lost by decreasing the amounts of sucrose present (Fig. 1A). Thus the uncoupling of electron transport and concomitant loss of photophosphorylation under these experimental conditions is correlated to the physical removal of the CF_1 part of the coupling factor complex leading to increasing proton permeability by exposing increasing amounts of the CF_0 proton channel. Full uncoupling of electron transport, and loss of photophosporylation, was obtained by removing about 50% of the CF_1, expressed either in terms of protein released or as Ca^{2+} ATPase activity. (Fig. 1A and B). Mechanical damage of the membranes caused by the freeze/thaw cycle as monitored by plastocyanin

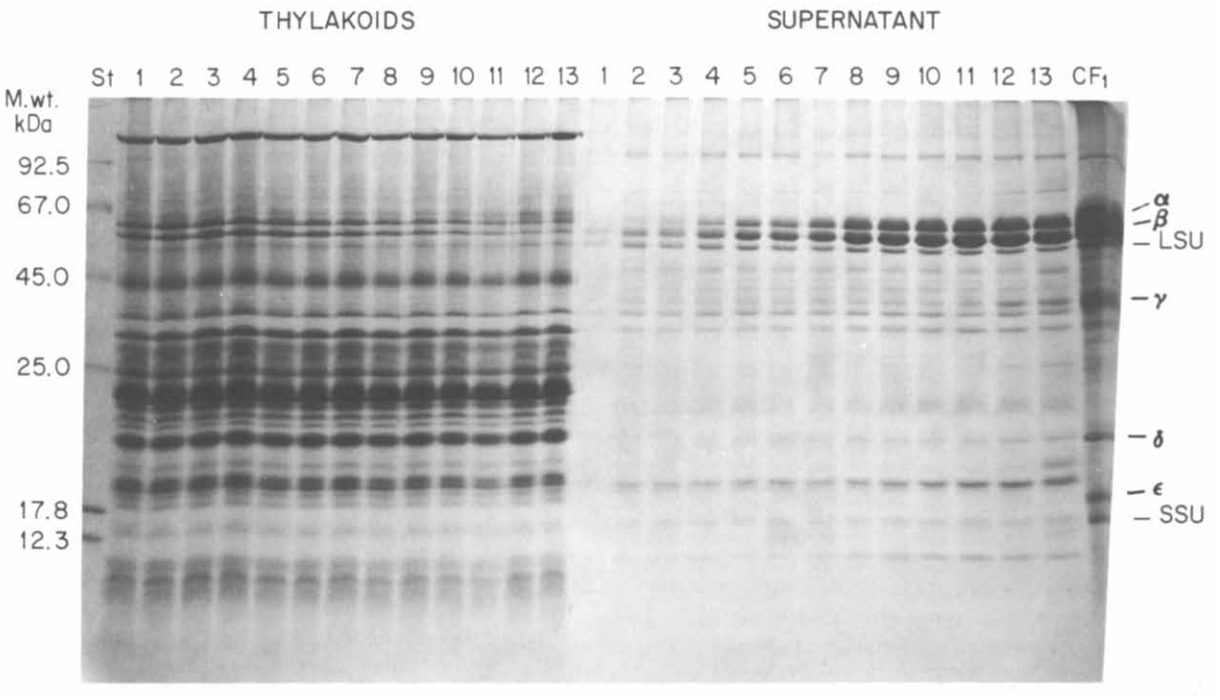

Fig. 2. SDS polyacrylamide gel analysis of thylakoids and supernatant after a 3 h, −20°C free/thaw regime. Lane 1, 0°C control; lanes 2–11, 100 mm NaCl, 250 mM or less sucrose as in Fig. 1; lanes 12 and 13, 250 mM and 500 mM NaCl. The molecular weight markers visible on the left-hand edge of the gel were phosphorylase B (92.5 kDa), bovine serum albumen (67.0 kDa), ovalbumen (45.0 kDa), chymotrypsinogen (25.0 kDa), myoglobin (17.8 kDa) and cytochrome *c* (12.3 kDa). Partially purified CF_1 was present on the right-hand edge of the gel showing the α (60 kDa), β(58 kDa), γ(37 kDa), δ(21 kDa) and ϵ(16 kDa) subunits the large (55 kDa) and small (12 kDa) subunits of ruBP carboxylase are also visible. After the freeze/thaw regime the vials were centrifuged (10 min, 12000 × *g*, 4°C), the supernatant decanted and recentrifuged, and the pellet resuspended to 500 µl in 0.2 M sucrose. 200 µl aliquots of supernatant were mixed with 200 µl of a 20% (w/v) solution of trichloroacetic acid, left on ice for 30 min and the precipitated protein pelleted by centrifugation (1 min, 12000 × *g*, 4°C). The supernatant was decanted and the pellet redissolved in 50 µl of 4% (w/v) SDS, 5% β-mercaptoethanol, 25 mM tris-HCl, 25% (v/v) glycerol, 0.01% bromophenol blue by sonication. Excess trichloroacetic acid was neutralized by the addition of 5 µl of 1 M tris-HCl (pH 8.0). 25 µl of the membrane suspension was mixed with 25 µl of the SDS solution and left for 10 min at room temperature. Samples were loaded onto a 15% (w/v) acrylamide/0.2% (w/v) bis acrylamide resolving gel, 5% (w/v) acrylamide/0.067% (w/v) bis acrylamide stacking gel and run overnight (15 h, 18 mA constant current) using the buffer system of Laemmli [41]. Gels were stained, fixed and destained with Coomassie brilliant blue R according to Piccioni et al. [28].

release and inhibition of linear electron transport was not a significant factor under those experimental conditions. The light-induced scattering changes measured at 90° and 180° (transmittance mode) were also abolished by increasing amounts of thylakoid uncoupling (Fig. 3), again in parallel to the abolition of photophosphorylation and proton gradient formation.

The ability of the uncoupled thylakoids to reconstitute light-dependent proton pumping by DCCD treatment was then investigated at two different pH values: 6.7, which is near the maximum for proton pumping; and 7.6, which is near the maximum for ΔpH formation and photophosphorylation. The uncoupling-dependent inhibition of proton pumping at pH 6.7 showed three phases (Fig. 4): (1) between 250 and 100 mM sucrose (10–50% CF_1 removal) about 60% of proton pumping was lost in parallel with the loss of photophosphorylation and the uncoupling of electron transport; (2) between 100 and 25 mM sucrose (50–70% CF_1 removal) the residual proton pumping remained relatively constant (75–125 µmol H^+/h per mg Chl); (3) 250 mM sucrose or less

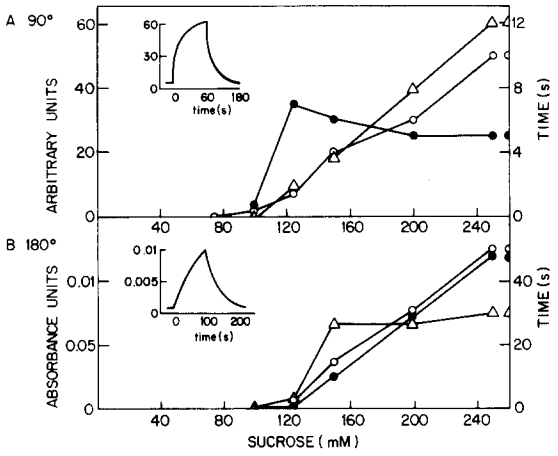

Fig. 3. The effect of freeze/thaw regimes (100 mM NaCl, up to 250 mM sucrose), 3 h, −20°C on (A) 90° scattering, (B) percentage transmittance changes at 540 nm. The medium (1.5 ml for A, 2.5 ml for B) contained 100 mM sorbitol, 10 mM $MgCl_2$, 10 mM Mes/KOH (pH 6.7), 20°C, 15 μg Chl·ml^{-1}, 0.1 mM methyl viologen, actinic light (610–800 nm) intensity was 40 W·m^{-2}. The insets show typical kinetic profiles of 90° scattering and 180° scattering changes over one light/dark period for a control sample. All measurements are the mean ± SD ($n = 5$). A. ○——○, 90° scattering extent ●——●, half-rise time, 90° scattering; △——△, half-dark decay time, 90° scattering. B. ○——○, 180° scattering extent; ●——●, half-dark decay time, 180° scattering; △——△, half-rise time, 180° scattering.

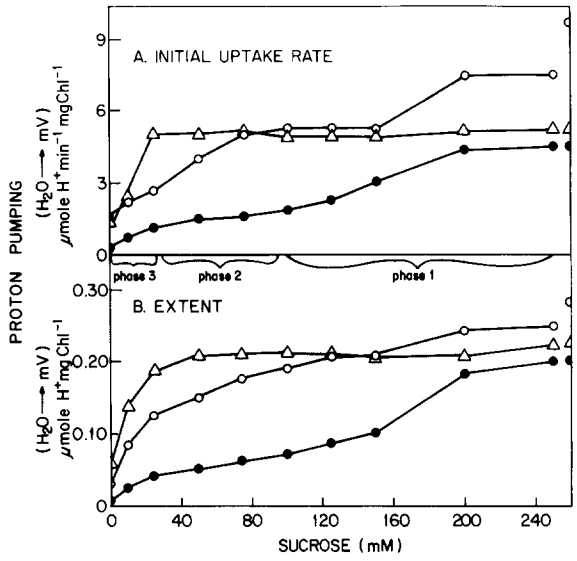

Fig. 4. The effect of freeze/thaw regimes (100 mM NaCl, up to 250 mM sucrose), 3 h, −20°C on proton pumping at pH 6.7 (initial uptake rate and total extent), and the ability of DCCD ($5 \cdot 10^{-5}$ M and 10^{-4} M) to reconstitute. Conditions as in Fig. 1A. The samples were preincubated with DCCD for 1 min in the dark before measurements were taken. All measurements are the mean ± SD ($n = 6$). (A) Initial uptake rate: ●——●, control; ○——○, reconstituted with $5 \cdot 10^{-5}$ M DCCD; △——△, reconstituted with 10^{-4} M DCCD; (B) Extent: ●——●, control; ○——○, reconstituted with $5 \cdot 10^{-5}$ M DCCD; △——△, reconstituted with 10^{-4} M DCCD.

(70–85% CF_1 removal) proton pumping was further inhibited (from 75 to $c \cdot 10$ μmol H^+/h per mg Chl) to a residual $c \cdot 5$% activity. The same general pattern of inhibition was observed at pH 7.6 (data not shown). The addition of 10^{-4} M DCCD resulted in complete reconstitution of proton pumping in phases 1 and 2, and substantial reconstitution in phase 3 ($c \cdot 10$% reconstitution when no sucrose was present to 100% reconstitution for 25 mM sucrose). Addition of $5 \cdot 10^{-5}$ M DCCD brought about a progressive reconstitution of proton pumping from 20–30% (absence of sucrose) to 100% (50–75 mM sucrose).

If proton pumping was reconstituted, then there should be a restoration of proton gradient formation. This was tested by measuring light-dependent 9-amino acridine fluorescence quenching at pH 7.6. It was only experimentally possible to use a maximal concentration of 10^{-5} M DCCD in the reconstitution experiments, as higher amounts abolished the light-dependent 9-amino acridine fluorescence quenching, presumably by direct chemical interaction, as DCCD is not an uncoupler of electron transport. The data presented in fig. 5 for the reconstitution experiments represent the maximal quenching obtained after a variable number of light (40 s)/dark (60 s) cycles as 10^{-5} M DCCD is suboptimal for the rapid reconstitution of proton pumping. In agreement with the results on the reconstitution of proton pumping, 9-amino acridine quenching was progressively restored between no sucrose ($c \cdot 10$% reconstitution) and 75 mM sucrose ($c \cdot 90$% reconstitution). Full reconstitution was obtained between 100 and 250 mM sucrose. The dark decay time was increased by DCCD reconstitution from 5 s for no sucrose present to a maximum of $c \cdot 11$ s for 10 mM sucrose. This is again in general agreement with the hypothesis of reducing the proton permeability of the membrane by this treatment.

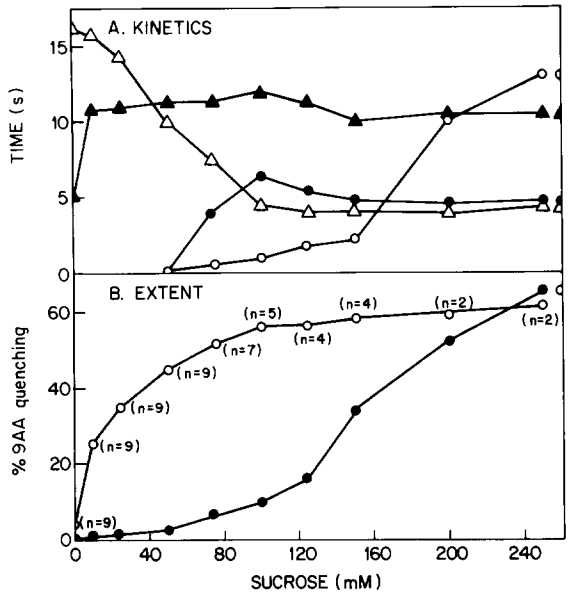

Fig. 5. The effect of freeze/thaw regimes (100 mM NaCl, up to 250 mM sucrose), 3 h, −20°C on light-dependent 9-amino acridine fluorescence quenching. Conditions as in Fig. 1B. 10^{-5} M DCCD was added for the reconstitution experiments and the samples subjected to a variable number of light (40 s)/dark (60 s) regimes (numbers in parentheses). All measurements are the mean ± SD ($n = 4$). (A) Kinetics: ●———●, half-rise time (s); (○———○, half-dark decay (s); △———△, half-rise time (s), $+10^{-5}$ M DCCD; ▲———▲, half-dark decay (s), $+10^{-5}$ M DCCD; (B) Extent: ●———●, control; ○———○, $+10^{-5}$ M DCCD.

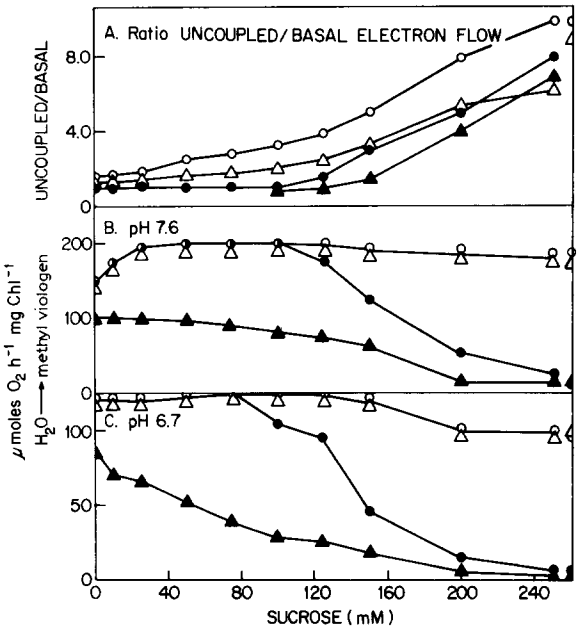

Fig. 6. The effect of freeze/thaw regimes (100 mM NaCl, up to 250 mM sucrose), 3 h, −20°C on linear electron transport ($H_2O \rightarrow$ methyl viologen at pH 6.7 and 7.6. The ability of $5 \cdot 10^{-5}$ M DCCD to reconstitute photosynthetic control. Samples were preincubated for 1 min in the dark with DCCD and/or 5 mM NH_4Cl before measurements were commenced. Conditions were as in Fig. 1. All measurements were the mean ± SD ($n = 4$). (A) Ratio uncoupled/basal electron transport: △———△, pH 6.7; ○———○, pH 7.6; ▲———▲, pH 6.7, $+5 \cdot 10^{-5}$ M DCCD; ●———●, pH 7.6, $+5 \cdot 10^{-5}$ M DCCD. (B) Electron transport (pH 7.6): ●———●, basal; ○———○, +5 mM NH_4Cl; ▲———▲, basal, $+5 \cdot 10^{-5}$ M DCCD; △———△, +5 mM NH_4Cl, $+5 \cdot 10^{-5}$ M DCCD. (C) Electron transport (pH 6.7): ●———●, basal; ○———○, +5 mM NH_4Cl; ▲———▲, basal, $+5 \cdot 10^{-5}$ M DCCD; △———△, +5 mM NH_4Cl, $+5 \cdot 10^{-5}$ M DCCD.

A further prediction if proton pumping and proton gradient formation were reconstituted in uncoupled thylakoids by DCCD treatment would be the restoration of some degree of photosynthetic control. The addition of $5 \cdot 10^{-5}$ M DCCD did indeed reduce the rates of linear electron transport of uncoupled thylakoids (Fig. 6). This could be reversed by the use of uncoupling agents, in this case 5 mM NH_4Cl, showing that electron transport itself was not affected. The ratio of uncoupled/basal electron transport in the reconstituted system increased from 1.5 (no sucrose present) to 4 (100 mM sucrose) at pH 7.6. The addition of 10^{-4} M DCCD gave qualitatively similar results, but at this high concentration it began to inhibit electron transport, and the ratio of uncoupled/basal flow was always less than at the lower DCCD concentration.

Finally, the reconstitution of light-dependent 90° scattering and the percentage of transmittance changes by $5 \cdot 10^{-5}$ M DCCD was examined at pH 6.7 (Figs. 7 and 8). The 90° scattering was completely reconstituted at 75 mM sucrose ($c \cdot 60\%$ CF_1 removal) and above. Partial reconstitution was obtained between no sucrose present ($c \cdot 15\%$ reconstitution, $c \cdot 85\%$ CF_1 removal) and 50 mM sucrose ($c \cdot 75\%$ reconstitution, $c \cdot 70\%$ CF_1 removal). The dark decay time was also increased by DCCD treatment from 4 s (no sucrose) to 12 s (50–250 mM sucrose). This is exactly in parallel to the reconstitution of proton pumping at pH 6.7 (Fig. 4). The percentage of transmittance changes,

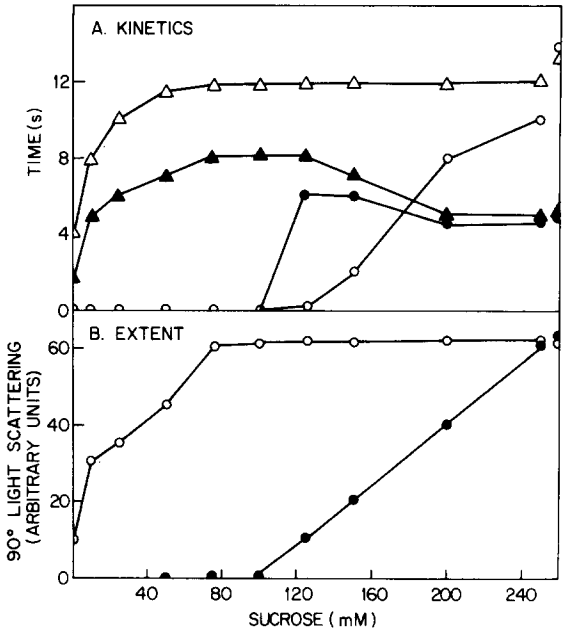

Fig. 7. The effect of freeze/thaw regimes (100 mM NaCl, up to 250 mM sucrose), 3 h, −20°C on 90° scattering. The ability of $5 \cdot 10^{-5}$ M DCCD to reconstitute. Conditions as in Fig. 3. The DCCD was added in the dark 1 min before the measurements were taken. All measurements were the mean ± SD ($n = 4$). (A) Kinetics: ●———●, half-rise time (s); ○———○, half-dark decay (s); ▲———▲, half-rise time (s), $+5 \cdot 10^{-5}$ M DCCD; △———△, half-dark decay (s), $+5 \cdot 10^{-5}$ M DCCD. (B) Extent: ●———●, control; ○———○, reconstituted with $5 \cdot 10^{-5}$ M DCCD.

in contrast, exhibited a much more variable response. No reconstitution was observed below 50 mM sucrose ($c \cdot 70\%$ CF_1 removal), and full reconstitution was only achieved at 200 mM sucrose ($c \cdot 15\%$ CF_1 removal).

Discussion

We have recently postulated [12,13] that the slow, light-induced scattering changes exhibited when thylakoids are continuously illuminated can be separated into two components. The well characterized 90° scattering response representing microconformational changes in membrane structure in response to proton uptake, and the percentage of transmittance changes representing slower and larger changes in membrane structure including changes in granal stacking. These two phenomena normally occur in parallel and only short-term glutaraldehyde treatment [12] separates these two processes.

In the present investigation, we have compared the DCCD dependent reconstitution of proton pumping of thylakoids physically uncoupled by the removal of varying amounts of CF_1 with the restoration of 90° scattering and percentage transmittance changes. It is known from previous work [7,9] that there is an ionic requirement for the scattering responses, which are maximal at pH values between 6 and 7, the same as for proton pumping [8]. 10 mM Mg^{2+}, a concentration that is saturating for these scattering responses [13], was employed in all experiments. The group of Steponkus et al. [24] has conducted experiments similar to ours. They have obtained ultrastructural evidence that, under their conditions, the thylakoid structure changes from the normal tubular to vesicular after the freeze/thaw regime both in uncoupled and protected thylakoids. The stock thylakoids we have used (1–2 mg Chl · ml^{-1} in 10 mM NaCl) were class D as defined by Hall [33]. These swollen membranes when placed in 100 mM sorbitol were able to restack on the addition of 10

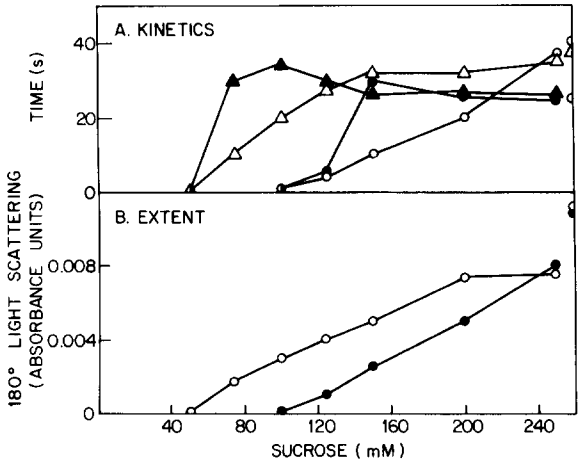

Fig. 8. The effect of freeze/thaw regimes (100 mM NaCl, up to 250 mM sucrose), 3 h, −20°C on percentage transmittance changes. The ability of $5 \cdot 10^{-5}$ M DCCD to reconstitute. Conditions as in Fig. 3. Samples were preincubated for 1 min in the dark before measurements commenced. All measurements were the mean ± SD ($n = 4$). (A) Kinetics: ●———●, half-rise time (s); ○———○, half-dark decay (s); ▲———▲, half-rise time (s), $+5 \cdot 10^{-5}$ M DCCD; △———△, half-dark decay (s), $+5 \cdot 10^{-5}$ M DCCD. (B) Extent: ●———●, control; ○———○, reconstituted with $5 \cdot 10^{-5}$ M DCCD.

mM $MgCl_2$, as monitored by the increase in the dark-scattering level. This measurement has been used by Diner and co-workers [34] in conjunction with ultrastructural evidence and chlorophyll fluorescence yield measurements as an indicator of the degree of thylakoid stacking. Unfortunately, we have no ultrastructural evidence yet of the thylakoid membrane conformation after the freeze/thaw regimes that we employed.

The division of freeze/thaw damage to proton pumping into three phases is in general agreement with the results of Steponkus et al. [24]. Their thylakoids only regained a maximum of 50% of control proton pumping values after DCCD reconstitution, possibly due to a degree of mechanical damage to the thylakoid membrane; as they have shown that plastocyanin was released from the thylakoid lumen under their experimental conditions which employed low osmotic-strength media (5 mM NaCl, up to 5 mM sucrose) and a slow ($2.8° \cdot h^{-1}$) freezing and thawing ($5.6°C \cdot h^{-1}$) regime. This supposition has been verified by recent results of Santarius and Giersch [35] and Hincha et al. [36,37]. We, in contrast, used higher osmotic and ionic strength conditions (100 mM NaCl, up to 25 mM sucrose), and a more rapid freeze ($c \cdot 0.5°C \cdot min^{-1}$)/thaw ($c \cdot 10°C \cdot min^{-1}$), and observed full reconstitution of proton pumping and no inhibition of electron transport.

The reconstitution of 90° scattering was fully in parallel to the reconstitution of proton pumping and thus again confirms the idea that this is a reflection of proton pumping. The slow percentage transmittance changes, however, did not show such a direct correlation to the reconstitution of proton pumping.

As first postulated by Barber and co-workers [37], one of the main factors involved in the stacking of thylakoids is the membrane surface charge. Mg^{2+} is the major ion which screens the membrane-bound fixed negative charges in vivo, and allows stacking to occur. There is a parallel requirement for Mg^{2+} for both granal stacking and the light-dependent scattering responses [13]. When thylakoids are freezed/damaged, among other physical alterations of the membrane properties is an irreversible membrane aggregation, indicating alterations in the surface charge density [38,39]. Grout et al. [39], by direct particle electrophoresis measurements showed that freezing damage reduced the surface charge density of whole *Chlamydomonas* cells. The irreversible alteration in membrane surface charge brought about by increasing freeze/thaw damage, thus preventing normal thylakoid restacking in the presence of Mg^{2+} or other ions would explain why the percentage transmittance changes were only partially reconstituted in our experiments, with no correlation to proton-pumping reconstitution.

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