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ELECTROSTATIC CONTROL OF CHLOROPLAST COUPLING FACTOR BINDING TO THYLAKOID MEMBRANES AS INDICATED BY CATION EFFECTS ON ELECTRON TRANSPORT AND RECONSTITUTION OF PHOTOPHOSPHORYLATION

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Summary

1. Increase in electron transport rate and the decay rate of the 518 nm absorption change, induced by EDTA treatment, is prevented by cations. The order of effectiveness is $C^{3+} > C^{2+} > C^{+}$.

2. In this respect methyl viologen is an effective divalent cation in addition to its action as an electron acceptor.

3. Complete cation irreversible EDTA-induced uncoupling occurs in the dark in 2 min. Light greatly stimulates the rate of uncoupling by EDTA. It is concluded that the uncoupling is due to release of coupling factor I from the thylakoid membrane.

4. Binding of purified coupling factor I to coupling factor I-depleted thylakoids can be achieved with any cation. The order of effectiveness is $C^{3+} > C^{2+} > C^{+}$, reconstituted thylakoids are active in photophosphorylation regardless of the cation used for coupling factor I binding.

5. The marked difference in the concentration requirements for cation effects on 9-aminoacridine fluorescence yield and for prevention of uncoupling by EDTA indicate that coupling factor I and its binding site have a lower surface charge density than the net surface charge density of the thylakoid membrane.

6. It is concluded that coupling factor I binding only occurs when negative charges on coupling factor I and its binding site are electrostatically screened by cations.

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; CF_1, coupling factor; PS, photosystem; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate.

7. Previously reported examples of uncoupling by low ionic conditions are discussed in relation to the basic concepts of diffuse electrical layer theory.

Introduction

Various phenomena have been shown to be affected by the cation composition of the diffuse layer associated with the negatively charged thylakoid membrane. These include changes in chlorophyll fluorescence yield [1], action of fluorescence probes [2,3] and control of thylakoid stacking [4]. Application of the Gouy-Chapman double-layer theory has allowed predictions of concentrations of cations at the thylakoid surface in relation to the bulk medium and also gives estimates of such parameters as the electrical field and space charge density at various places in the diffuse layer [1,4]. An important feature of these considerations is that the effectiveness of cations for screening fixed negative surface charges depends on the valency of the cation and not its chemical nature or its co-ion. The order of effectiveness is $C^{3+} > C^{2+} > C^{+}$.

Over the years there have been many reports of cation effects on electron transport and phosphorylation in isolated thylakoids. In many cases a differential effect of monovalent and divalent cations had been noted [5—9] but no interpretation was made in terms of the electrostatic theory mentioned above. The same type of differential effect of cations had also been observed for chlorophyll fluorescence yield [10,11], thylakoid stacking [12] and spillover of excitation of energy from Photosystem II (PS II) to Photosystem I (PS I) [10]. Coupled with these observations many workers have noted various effects of EDTA on photosynthetic processes which too may be associated with modification of the cation composition in the layer adjacent to the membrane surface [5—7,13].

Jagendorf and Smith [5] originally showed that treatment of thylakoid membranes with EDTA in a medium of low ionic strength induces uncoupling of photosynthetic electron flow. Most significantly they found that uncoupling was prevented by the presence of cations, monovalents being effective at a 10-fold higher concentration range than divalents. It has since been demonstrated that uncoupling by EDTA treatment in a low ionic medium is due to the removal of the soluble coupling factor (CF_1) portion of the ATP synthetase complex [14] leaving a membrane-bound component which is permeable to protons [6,15—17]. This type of uncoupling has also been detected by an increase in the decay rate of the flash-induced 518 nm absorbance change [16,17].

The action of EDTA and also of pyrophosphate buffer [18] in CF_1 removal from thylakoid membranes is presumably due to chelation of divalent cations necessary for CF_1 binding. However, the protective effect of monovalent cations suggests that CF_1 binding may be controlled by some electrostatic screening process and seems to rule out a model in which a particular divalent cation (presumably Mg^{2+} in vivo) functions as a 'bridge' to hold CF_1 onto its binding site(s) on the membrane (see Refs. 15 and 19). Some support for the electrostatic control of CF_1 binding comes from a brief mention in the liter-

ature that monovalent cations can partially replace Mg^{2+} in the recoupling of CF_1 to CF_1-depleted membranes [20].

Jagendorf and Smith [5] and others [7,8] showed that uncoupling could be induced without EDTA treatment if thylakoids were washed in water or suspended in low salt-containing media. Walz et al. [8] found a particularly striking effect when chloroplasts were suspended in a glycine medium containing very few cations. They found that no uncoupling occurred in this medium unless a low level of monovalent cations (2—4 mM) was added. Additions of monovalent cations at higher levels (greater than 10 mM) or the addition of low levels of divalent cations (greater than 0.2 mM) prevented the uncoupling of electron transport. Again it was noted that the cation effect was independent of the species used within a particular valency group and that divalent cations were at least ten times more effective than monovalent cations at maintaining the coupled rate.

As pointed out previously [4] the antagonism between the effect of divalent and monovalent cations or monovalent cations at low and high concentrations on electron transport [8] is similar to that seen with cation-induced chlorophyll fluorescence changes [21]. The only difference between the two cation-induced phenomena is that the effect on electron transport occurs at a lower concentration range than the effect on chlorophyll fluorescence. Nevertheless, Barber et al. [4] suggested that both phenomena are controlled by the positive space charge density immediately adjacent to the membrane surface (i.e. to electrostatic screening).

It has been argued that the high chlorophyll fluorescence yield observed with thylakoids isolated in essentially cation-free medium is mainly due to retention of Mg^{2+} carried over with the membranes [22]. Treatment with EDTA removes the surface Mg^{2+} and lowers the fluorescence yield [23] as does the addition of low levels of monovalent cations [22].

In this paper we have reinvestigated the actions of cations and EDTA on photosynthetic electron flow especially in relation to control of electron transport rate by CF_1 binding and CF_1 binding to membranes depleted of this complex. Our work emphasizes the importance of electrostatic effects at the membrane surface and gives a basis for reinterpretation of some of the earlier data reported by the various workers mentioned above.

Methods

Chloroplasts were isolated from 7 to 10-day-old pea plants in an ice-cold grinding medium consisting of 0.4 M sucrose, 0.05 M Tris-HCl buffer, 10 mM NaCl and 10 mM sodium isoascorbate, pH 7.8. The ratio of leaf to grinding medium was 1 g leaf : 3 ml medium. The slurry was filtered through ten layers of muslin plus a thin layer of non-absorbant cotton wool and centrifuged at $2200 \times g$ for 30 s (total centrifugation time being approx. 90 s). The supernatant was discarded carefully so that the large sloppy pellet was retained. The long grinding time (approx. 10 s with a Polytron), presence of monovalent cations in the grinding medium and retention of the sloppy pellet, ensured that the majority of the chloroplasts isolated had lost their outer membranes (see Ref. 24). The pellet was resuspended in a small volume of grinding medium

minus sodium isoascorbate such that the chlorophyll concentration, measured by the method of Arnon [25] was always greater that 2.5 mg · ml^{-1}.

Oxygen evolution and uptake was measured with a Clark-type oxygen electrode [26]. Illumination was with red light of about 300 J · m^{-2} · s^{-1}. 9-Aminoacridine fluorescence was measured as described previously [2]. Flash-induced 518-nm absorption changes were measured at room temperature with a rapid kinetic apparatus. Excitation was brought about by single 10-μs flashes from a xenon lamp which were passed through a Schott 2 mm RG665 cut-off filter. The intensity of the 518 nm measuring beam was 0.72 J · m^{-2} · s^{-1} and it was detected with an EMI 9555B photomultiplier protected by a Schott 2 mm BG18 cut-off filter. Signals were recorded using a Datalab DL905 transient recorder.

For experiments on recoupling, market spinach was ground in a medium containing 0.33 M sorbitol, 0.05 M *N*-tris(hydroxymethyl)methylglycine (Tricine) brought to pH 7.8 with NaOH, 2 mM sodium isoascorbate, 0.2 mM $MgCl_2$ and 0.2 mg · ml^{-1} bovine serum albumin. After centrifuging (5000 rev./min in a Sorvall centrifuge for 5 min) the chloroplasts were resuspended and washed once in the same buffer. The procedures for uncoupling and recoupling were modified from those of Hesse et al. [27]. The chloroplasts were centrifuged, resuspended and washed three times in buffer containing 10 mM sodium pyrophosphate brought to pH 7.5 with HCl, 1 mM sodium isoascorbate and 0.2 mg · ml^{-1} bovine serum albumin. After the last wash they were resuspended at 0.05 mg chlorophyll · ml^{-1} in 0.33 M sorbitol, 2 mM Tricine brought to pH 7.6 with Tris base, 1 mM dithiothreitol, 0.1 mg · ml^{-1} bovine serum albumin and 5 μm $CaCl_2$. (The concentration of $CaCl_2$ needed to prepare thylakoids that were fully uncoupled but able to be recoupled with CF_1 varied from preparation to preparation; it was re-tested with each new preparation.) The thylakoid membranes were maintained at 0—4°C in this medium for 20 min, then uncoupling was stopped by adding 1/10 vol. of 0.2 M $CaCl_2$, 0.1 M Tricine at pH 7.5. The thylakoid membranes were centrifuged, then resuspended at 2.0 mg chlorophyll · ml^{-1} in a medium used for frozen storage, modified from the one suggested by Cohen [28] and containing 50% glycerol, 5% Me_2SO, 10 mM NaCl, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate (Hepes) buffer at pH 7.5, and 20 mg · ml^{-1} of bovine serum albumin. These membranes could be used after storage in a liquid N_2 refrigerator for up to 6 months.

When the uncoupled chloroplasts were required a tube was thawed, and to it were added 2 vols. of 0.3 M sucrose, 2 mM Tricine (sodium salt), 1 mg · ml^{-1} bovine serum albumin and 1 mM dithiothreitol, all at pH 7.6. The membranes were sedimented and resuspended in fresh sucrose/Tricine/bovine serum albumin/dithiothreitol medium at 1 mg chlorophyll · ml^{-1}.

CF_1 was prepared according to Binder et al. [29] and stored as an $(NH_4)_2SO_4$ precipitate. Prior to use it was centrifuged, redissolved and dialyzed in a medium containing 10 mM Tricine (sodium salt), 0.5 mM ATP and 3.0 mM sodium azide, pH 7.6. The recoupling reaction was performed in a volume of 0.2 ml by mixing 0.1 ml of the chloroplasts, 40 μl of CF_1, 20 μl of a 5-fold concentrated solution similar to the one the chloroplasts were in but lacking Tricine (i.e. 1.5 M sucrose, 5 mM dithiothreitol, 5 mg · ml^{-1} bovine serum

albumin and 40 μl of either H_2O or a 5-fold concentration of the salt to be tested. The mixture was incubated at room temperature for 5 min, then diluted 10-fold by adding 1.8 ml of an ice-cold solution containing 0.3 M sucrose, 1 mM dithiothreitol, 1 mg · ml^{-1} bovine serum albumin and the salt being tested for recoupling activity for each tube. These thylakoid membranes were centrifuged for 5 min at 5000 rev./min in a Sorvall centrifuge and the supernatant was discarded. The membranes were resuspended by adding 0.36 ml of 0.3 M sorbitol, 20 mM NaCl and 1 mg · ml^{-1} bovine serum albumin. The chlorophyll concentration at this point, due to losses in centrifuging and resuspending, was between 0.15 and 0.20 mg · ml^{-1}; it was measured in each tube by extracting an aliquot of 0.1 ml with 0.9 ml of 80% acetone [25]. Photophosphorylation was assayed using 0.1 ml aliquots of chloroplasts added to 0.9 ml of medium as described earlier [30], with the non-esterified phosphate at the end of the reaction removed by precipitation of the phosphomolybdate complex with triethylamine [31].

Tris(ethylenediamine)cobaltic trichloride was obtained from Alfa Products, Danvers, MA.

Results

Initially we studied the requirements for uncoupling of pea thylakoid electron transport by low-salt conditions. Table I shows that it was necessary to add a low level of EDTA to stimulate the rate of ferricyanide reduction significantly. Unlike Walz et al. [8] we found that low levels of monovalent cations (1.5—4.0 mM K^+) had little effect on the rate of electron transport, although our pea chloroplasts were isolated and assayed under very similar conditions to those employed by Walz et al. [8] for lettuce chloroplasts. However, we did find, like Walz et al. [8], that increasing the level of monovalent cations prevented the EDTA-induced uncoupling (Table I).

Fig. 1 shows that the decay rate of the flash-induced 518 nm absorbance change was also markedly stimulated by addition of EDTA and that either 20 mM KCl or 0.2 mM $MgCl_2$ added immediately after the EDTA prevented this increase. This indicates that uncoupling by EDTA is due to an increase in

TABLE I

EFFECT OF KCl ON EDTA UNCOUPLING OF FERRICYANIDE-DEPENDENT OXYGEN EVOLUTION

The reaction medium contained: 26 μg chlorophyll · ml^{-1}, 40 mM glycine (pH 7.6 with KOH) and 0.3 mM potassium ferricyanide. KCl, 0.3 mM EDTA and 1.3 · 10^{-7} M nigericin were added in the order indicated before the onset of illumination. The background K^+ level was approx. 1.5 mM.

	μatoms oxygen · mg^{-1} chlorophyll · h^{-1}
No additions	29
+ 2.5 mM KCl	30
+ EDTA	284
+ EDTA + 2.5 mM KCl	287
+ EDTA + 30 mM KCl	39
+ EDTA + 30 mM KCl + nigericin	486

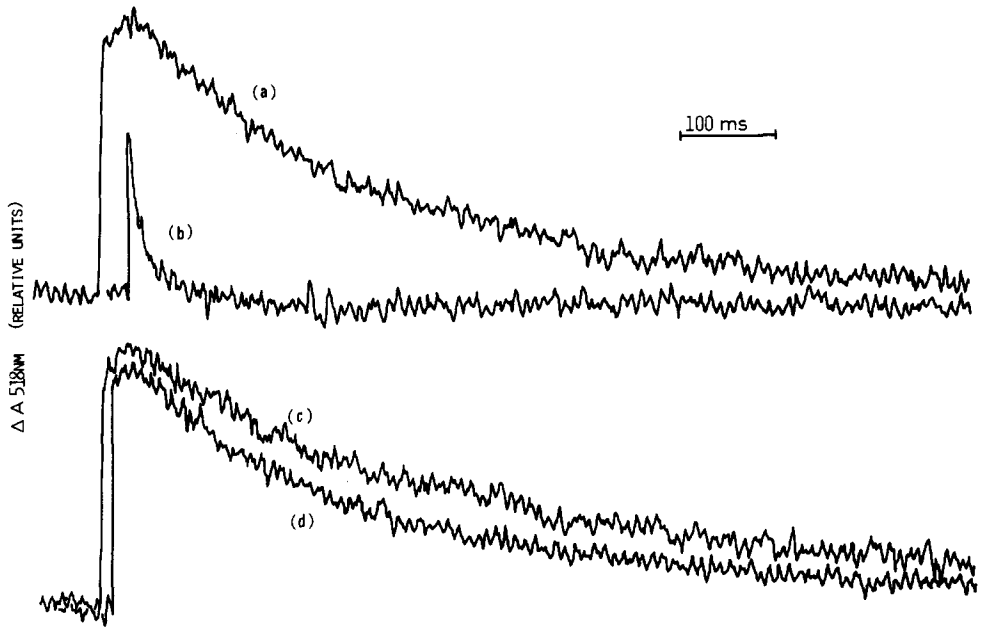

Fig. 1. Effect of EDTA treatment on the rate of decay of the 518 nm absorption change. The reaction medium contained: 28 μg chlorophyll · ml^{-1}, 60 mM glycine (pH 7.6 with less than 0.2 mM KOH) and 0.3 mM $K_3Fe(CN)_6$. (a) No additions; (b) plus EDTA alone; (c) plus EDTA and 0.2 mM $MgCl_2$, and (d) plus EDTA and 20 mM KCl. EDTA concentration was 50 μM. $MgCl_2$ and KCl were added 15 s after EDTA and all reaction mixtures were incubated for 4 min in the dark before flash excitation.

the conductance of the thylakoid membrane assuming that the flash-induced signal is acting as a molecular voltmeter as argued by Witt and colleagues [32]. Thus it seems that the low electrical conductance of thylakoid membranes suspended in a low-salt medium is due to the presence of residual divalent cations at the membrane surface which are removed by addition of EDTA.

The fact that low levels of divalent (Mg^{2+}) and higher levels of monovalent (K^+) cations inhibit the action of EDTA is indicative of an electrostatic mechanism [1,4]. According to electrostatic theory, trivalent cations should be more effective than di- or monovalent cations at preventing uncoupling by EDTA. This is borne out by the experiment shown in Fig. 2a. In this experiment the trivalent, tris(ethylenediamine)cobaltic cation was used since its structure prevents it from chelating with EDTA or binding to the chloroplast membrane [33], a problem often encountered with other trivalent cations such as La^{3+} [34]. However, it should be noted (Fig. 2a) that the curve for Mg^{2+} is due to its titration with EDTA, an effect overcome by using methyl viologen as a divalent cation for alleviating the uncoupling action of EDTA (see Fig. 2b). In this experiment methyl viologen also acted as the electron acceptor, electron transport being measured as an uptake of oxygen. At concentrations of methyl viologen below 10 μM, EDTA brought about uncoupling in the usual way but with higher concentrations of the acceptor no uncoupling effect was observed. The concentration of di- and monovalent cations required to prevent EDTA-induced uncoupling are similar to those found by others

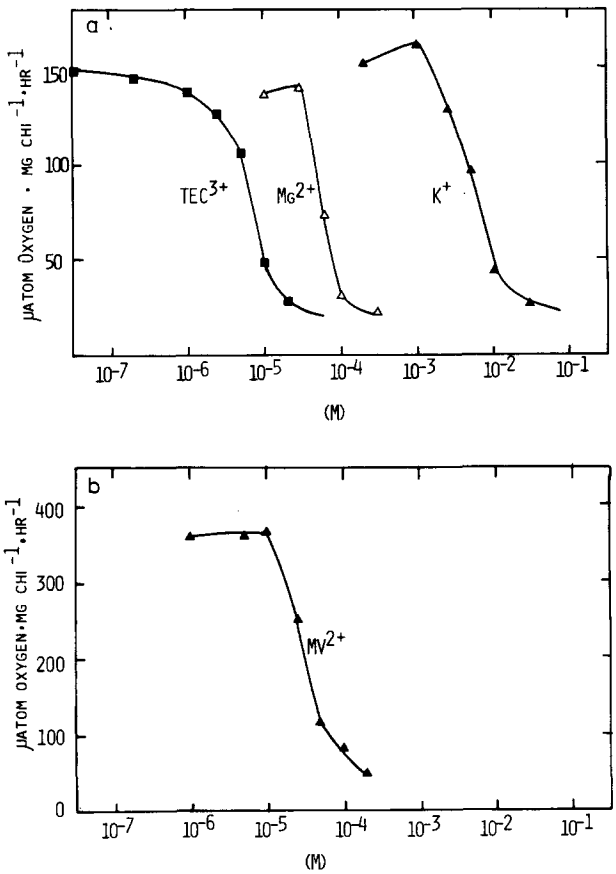

Fig. 2. (a) Effect of cations of different valency on ferricyanide-dependent oxygen evolution rates stimulated by EDTA. The reaction medium contained: 20 μg chlorophyll · ml^{-1}, 60 mM glycine (pH 7.6 with less than 0.2 mM KOH), 0.6 mM $K_3Fe(CN)_6$ and 0.1 mM EDTA. ■, tris(ethylenediamine)cobaltic trichloride (TEC^{3+}); △, $MgCl_2$, and ▲, KCl. Cations were added 15 s after addition of EDTA. (b) Effect of methyl viologen (MV^{2+}) concentration on oxygen uptake rates stimulated by EDTA. The reaction medium contained: 25 μg chlorophyll · ml^{-1}, 60 mM glycine (pH 7.6 with less than 0.2 mM KOH) and 0.6 mM EDTA. Methyl viologen and 0.1 mM sodium azide were added 15 s after EDTA.

[5,7,8] although in some cases the uncoupling was brought about simply by low ionic conditions.

Thus it appears that uncoupling by low-salt conditions either in the presence or absence of EDTA may be attributed to the same electrostatic mechanism. As EDTA treatment at low ionic strength is known to remove CF_1 [14] from the thylakoid membrane it seems likely that CF_1 binding involves some form of electrostatic phenomenon which is most likely associated with cation screening of surface negative charges. Thus it seems likely that the addition of suitable screening cations to the medium reduces the coulombic repulsion between charged regions of the CF_1 protein surface and its binding site on the thylakoid membrane.

We therefore investigated the time course of uncoupling and found that although cations prevented uncoupling if added immediately after EDTA they did not 'recouple' electron flow if added after the onset of illumination. A

more detailed investigation showed that under dark conditions it took 2 min of preincubation with EDTA before the addition of cations was totally ineffective at reversing uncoupling (see Fig. 3). However, it can be seen that light accelerates the rate of uncoupling as it was essentially complete on illumination in the absence of cations after the briefest dark incubation time tested. The electron transport rate was measured within 30 s of the onset of illumination.

The data presented here indicate that CF_1 is electrostatically held to its binding site on the thylakoid surface. To prove this point further we have conducted reconstitution experiments by adding back purified CF_1 to CF_1-depleted thylakoids under different ionic conditions and determining their capacity to carry out photophosphorylation (see Table II).

After the incubation of CF_1-depleted thylakoids, CF_1 and the various salts (see Methods) it was necessary to dilute the mixtures 10-fold to stop further reassociation of CF_1 with the membranes. The thylakoids were then sedimented and resuspended in a standard buffer containing 20 mM NaCl to prevent further or renewed uncoupling from occurring. This procedure removed most of the salt being tested, to avoid possible direct interference in the phosphorylation reactions. It also removed free CF_1 so that further recoupling would not occur due to Mg^{2+} present as a component of the photophosphorylation reaction mixture. The observed rates of ATP synthesis were thus an assay for CF_1 which had reassociated with the depleted membranes, and remained bound throughout the dilution and centrifugation steps.

No specificity was observed for cations functioning in this reassociation (Table II). Effective species included both organic and inorganic cations, and the various anions used (Cl^-, SO_4^{2-}, acetate, Tricine (sodium salt), glucuronate, citrate) had no perceptible influence. That the cations were the effective species is indicated from the widely differing concentrations required, depen-

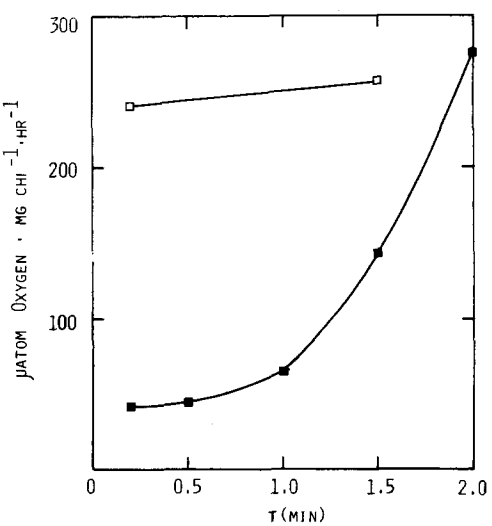

Fig. 3. Time course for uncoupling of methyl viologen-dependent oxygen uptake by EDTA. Thylakoids were incubated in the dark in a reaction medium as described for Fig. 2b. The methyl viologen concentration was 5 μM. After incubation for the times indicated the reaction mixtures were illuminated either immediately (□) or after addition of 20 mM KCl plus 1 min incubation in the dark (■).

TABLE II

LACK OF CATION SPECIFICITY IN RECOUPLING SPINACH CHLOROPLASTS

The rate is the phosphorylation rate = μmol ATP · mg^{-1} chlorophyll^{-1} · h^{-1}.

Divalent cations (3 mM)	Rate	Sodium salts (20 mM)	Rate	Other monovalent cations (20 mM)	Rate
—	0	NaCl	144	LiCl	142
$MgCl_2$	129	Na_2SO_4	104	KCl	97
$BaCl_2$	124	Sodium acetate	77	*N*-Ethylmorpholine-HCl	108
$CaCl_2$	46	Sodium citrate	114	Tris-HCl	75
$CoCl_2$	62	Sodium glycinate	96	Choline-HCl	152
Spermidine-HCl	63	Tricine (sodium salt)	120	Ethanolamine-HCl	142
		Sodium glucuronate	99	Tris/glucuronate	133
				Lysine-HCl	131

dent on the cation valency (Figs. 4 and 5). An optimum concentration for Na^+ occurred at 15—20 mM, For Mg^{2+} at 1—3 mM for tris(ethylenediamine)-cobaltic trichloride at 0.2—0.3 mM.

In other experiments CF_1 was labelled with [^{3}H]acetic anhydride, and reassociation of the label with depleted membranes was measured after sedimenting through a silicone oil layer as in Ref. 35. This physical reassociation showed a requirement for cations, with concentration dependence for NaCl, $MgCl_2$ and tris(ethylenediamine)cobaltic trichloride very similar to that found for reconstituting photophosphorylation (data not shown).

It was conceivable that NaCl might have acted by causing the release of bound or internal Mg^{2+} in the thylakoid membranes on an ion-exchange principal, and the effective species might have been the released Mg^{2+}. However, as the sodium salt of EDTA was fully effective (Fig. 4) this possibility can be ruled out.

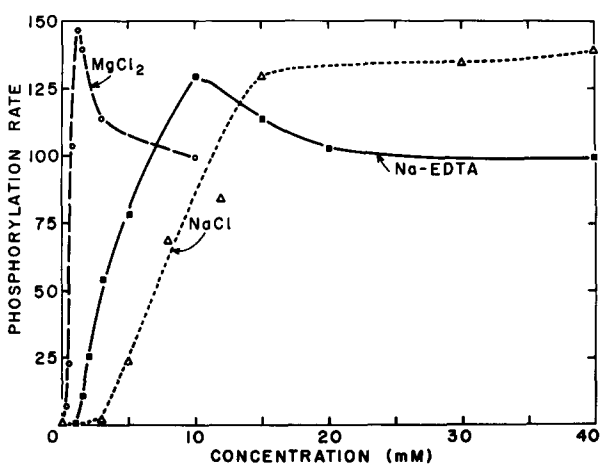

Fig. 4. Recoupling of depleted spinach thylakoid membranes by NaCl, sodium EDTA or $MgCl_2$. Procedures used are described in Methods. The concentration of EDTA used is that of the anion; hence at pH 7.6 sodium concentrations are 2—3 times higher.

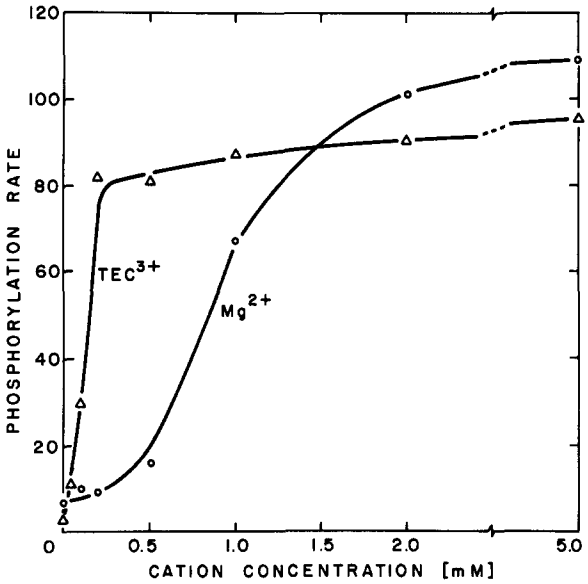

Fig. 5. Comparative effectiveness of $MgCl_2$ and of tris(ethylenediamine)cobaltic trichloride (TEC^{3+}) in permitting recoupling of spinach thylakoid membranes.

If the cations had some sort of specific chemical function in the binding process, use of a monovalent instead of a divalent cation might lead to a weaker bond between CF_1 and its binding site. To test this concept recoupling mixtures containing the same amounts of CF_1 and chlorophyll (100 μg of each) were diluted to varying extents, keeping the ionic composition constant (Fig. 6). After incubation the mixtures were all made up to the same large volume (2.0 ml), centrifuged and used for phosphorylation as usual. As might be expected for a binary reaction, recoupling was very sensitive to the concentrations of the reassociating species. However, there was no sign of any difference

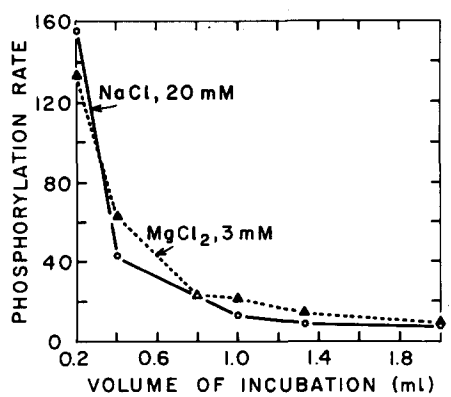

Fig. 6. Dependence of recoupling on the concentrations of chloroplasts and CF_1. All tubes contained 100 μg each of chlorophyll and of CF_1 so that the concentrations of each were 0.5 mg $\cdot$ ml^{-1} in the 0.2 ml reaction mixture. The ionic composition was the same in all tubes; after the 5 min incubation all tubes were made up to 2.0 ml with the standard medium (see Methods) containing either 20 mM NaCl or 3 mM $MgCl_2$, as present during the original incubation. For further details see text.

TABLE III

COMPARISON OF THE CONCENTRATIONS OF MONOVALENT AND DIVALENT CATIONS REQUIRED FOR HALF-MAXIMAL EFFECT ON RELEASE OF CHLOROPLAST QUENCHING OF 9-AMINOACRIDINE FLUORESCENCE (9-AA Fϕ), COUPLING OF ELECTRON TRANSPORT AND DECAY RATE OF THE 518 nm ABSORBANCE CHANGE

Reaction media were as described in Fig. 1 except that for 9-aminoacridine fluorescence yield changes 10 μM 3-(3',4'-dichlorophenyl)-1,1-dimethylurea and 25 μM 9-aminoacridine were also present. Monovalent cation was K^+ and divalent cations were Mg^{2+} or the methyl viologen cation.

Valency of cations	$C_{1/2}$ (M)		
	9-AA Fϕ	Coupling	Decay of ΔA_{518}
Monovalent	$2.0 \cdot 10^{-2}$	$3.2 \cdot 10^{-3}$	$3.4 \cdot 10^{-3}$
Divalent	$1.4 \cdot 10^{-4}$	$3.0 \cdot 10^{-5}$	$2.4 \cdot 10^{-5}$

in the concentration response, indicating no difference in binding affinities, when NaCl was used in place of $MgCl_2$ (Fig. 6).

It should be noted that higher concentrations of cations are required for this type of reconstitution than are required for the prevention of EDTA-induced coupling. Nevertheless in both cases the concentration ranges involved are significantly lower than those associated with more general thylakoid electrostatic effects such as cation-induced changes in chlorophyll fluorescence [10,11], thylakoid stacking [12] and cation-induced release of 9-aminoacridine or 2-*p*-toluidinonaphthalene-6-sulphonate fluorescence quenching [2,3]. This is emphasized in Table III where the concentrations of cations needed for release of 9-aminoacridine quenching are compared with the levels necessary to prevent EDTA-induced uncoupling monitored either as electron transport or rate of decay of the flash-induced 518 nm absorption change. Such a difference indicates that the surface charge densities associated with CF_1 binding are substantially lower than for the average surface charge density of the thylakoid membrane [36].

Discussion

The data reported in this paper confirm and extend the reports of others [5,6,8] of nonspecific cation control of photosynthetic electron transport. We argue that this control involves changes in the ionic composition of the diffuse layer adjacent to the thylakoid surface and that in particular it is closely related to variations in CF_1 binding to its specific membrane site. We conclude that the binding of CF_1 to its membrane site requires electrostatic screening (or possibly neutralisation) of surface negative charges. In the past the fact that CF_1 is removed by EDTA treatment has led to the suggestion that Mg^{2+} has some kind of bridging function in the binding of CF_1 to the membrane [15]. Although reconstitution of photophosphorylation is normally carried out with Mg^{2+}, Ca^{2+} has been demonstrated to bring about binding of CF_1 to CF_1-depleted thylakoids [37] and as already mentioned in Introduction Shoshan and Shavit [20] reported partial reconstitution with K^+. We have demonstrated that a number of different cations will reconstitute photophos-

phorylation and that the order of effectiveness is dependent only on valency and not on the specific nature of the cation. All this is in line with established electrostatic theory (see Ref. 4).

There is much evidence that in vivo Mg^{2+} is the major cation in the diffuse electrical layer adjacent to the thylakoid membrane [22] and it would not seem unreasonable to assume that this cation is normally responsible for the CF_1-membrane binding. Certainly the ability of sodium EDTA to remove CF_1 would be in agreement with this but clearly in vitro other cations can substitute for Mg^{2+}. Thus CF_1 release from the membrane can be envisaged as follows. On addition of EDTA to thylakoids incubated in a low-cation medium (less than 1 mM monovalent cation only), divalent cations in the diffuse layer and adsorbed onto the membrane surface are chelated and monovalent cations (associated with the EDTA added and from the bulk medium) take over as the major screening cation. However, at this low bulk concentration of monovalent cations the positive space charge density at the membrane surface would be lowered as explained by Barber et al. [4]. A lowering of this space charge density parameter results in a decrease in electrostatic screening and a concomitant increase in coulombic repulsion between the fixed negative charges associated with the surfaces of the CF_1 and of the membrane binding site. From the curve shown in Fig. 3 the rate of diffusion of CF_1 away from the thylakoid surface is relatively slow under dark conditions so that when the bulk cation concentration is raised ($C^{3+} > 2 \cdot 10^{-5}$ M, $C^{2+} > 2 \cdot 10^{-4}$ M, $C^{+} > 3 \cdot 10^{-2}$ M) within 45—60 s of the EDTA treatment, the resulting electrostatic screening allows the CF_1 to reassociate with its specific membrane site. Apparently this reassociation cannot occur after longer incubation times, the CF_1 having diffused too far from the membrane surface. Under these circumstances the level of CF_1 has to be artificially raised in the bulk medium to allow reconstitution to occur. The stimulation by light of the rate of removal of CF_1 suggests that a conformational change takes place, which may for example, expose more negative charges and hence increase the force of repulsion between CF_1 and its binding site.

Reconstitution of an active ATP synthetase complex by adding CF_1 to CF_1-depleted thylakoids requires the presence of cations, but again there is no specificity except that the degree of effectiveness depends on the valency. However, the concentration range of cations required was higher than that required to prevent the removal of CF_1 by EDTA treatment. This is probably due to the need to screen the additional charges on the membrane surface as a whole, hence lowering the surface potential and allowing the negatively charged CF_1 to approach its binding site.

Applications of the diffuse layer theory of Gouy-Chapman has given an explanation for the antagonistic effect of divalent and monovalent cations on chlorophyll fluorescence and stacking [4]. Essentially it has been shown that when a negatively charged surface with divalent cations as the main species in the diffuse layer is suspended in a medium of very low cation concentration the positive space charge density adjacent to the surface remains high. As the bulk monovalent cation concentration is increased a critical monovalent to divalent ratio is reached and the positive charge density decreases to a minimum value. Thus the effect is to decrease electrostatic screening and enhance coulombic

repulsion. In the case of the thylakoids, unstacking occurs and chlorophyll fluorescence is brought to a minimum. Good electrostatic screening due to an increase in the diffusible positive charge at the surface, can be reestablished by introducing low levels of divalent or trivalent cations [33] or high levels of monovalent cations. Under these conditions thylakoid membranes restack and chlorophyll fluorescence is returned to the high level again. In the experiment reported above it was necessary to remove surface divalents, presumably Mg^{2+}, with EDTA so as to allow sufficient coulombic repulsion to occur between the CF_1 protein and the membrane surface. In the case of membrane unstacking or the decrease in chlorophyll fluorescence, the introduction of monovalent cations is adequate to bring about the same effect.

Walz et al. [8] were able to bring about the uncoupling effect without using EDTA by simply adding low levels of monovalent cations to a cation-free medium (lettuce chloroplasts). To reinstate coupled electron flow they found, as we have, that it was necessary to add back ranges of divalent or monovalent cations in much lower concentrations than those needed for thylakoid stacking [38] or for the cation-induced increase in chlorophyll fluorescence [1]. This finding indicates that the surface charge density associated with CF_1 and its binding site is lower than the average surface charge density of the thylakoid membrane.

As stated above, in order to uncouple, a critical monovalent to divalent cation ratio must be reached, at which point the positive space charge density will be at a minimum value. The difference between our experimental results and those of Walz et al. [8] may be due to the presence of higher background levels of divalent cation in our preparations (pea chloroplasts). We usually found a synergistic effect of EDTA and monovalent cations suggesting that when the divalent concentration was lowered the addition of monovalents could displace those remaining divalents in the diffuse layer.

This new interpretation of low ionic strength-induced uncoupling in chloroplasts resolves several questions which have been raised in the literature (see Refs. 14 and 19). Mg^{2+} cannot be performing some kind of bridging function in CF_1 binding [15] because even after replacement with cations of different valency photophosphorylation can still take place. However, it should be emphasized that in vivo magnesium is probably the screening cation associated with the CF_1 binding site.

It has also been noted that removal of CF_1 is accomplished in media of low ionic strength. As these conditions bring about numerous changes in thylakoid structure including unstacking it has been suggested that proton leakage may not only be via the 'hole' through the membrane component of the ATP synthetase [19] but that other areas of the membrane may have become permeable to protons. However, we found that though removal of CF_1, and and hence uncoupling, was brought about under conditions that would also induce unstacking, uncoupling was completely prevented by concentrations of monovalent cations (approx. 10 mM K^+) which are well below those required to bring about stacking [4]. Hence it seems likely that uncoupling is in fact due solely to leakage of protons through the CF_0.

Finally it should be mentioned that, although EDTA treatment completely uncouples electron transport by removal of CF_1 from its specific site on the

thylakoid surface, the effect is incomplete with 30—50% of the CF_1 remaining bound to the surface [19]. Apparently the loss of significant portions of CF_1 exposes sufficient CF_0 to release control of electron transport. The data and arguments presented here suggest that the incomplete removal of CF_1 may reflect the background level of monovalent cations, due to those present in the medium and to those added with EDTA. That is the removal of divalent cations from the surface is partially compensated for by monovalent cations entering the diffuse layer. Indeed, as shown here and by Jagendorf and Smith [5] there is a dual effect of EDTA itself. At low concentrations it efficiently removed CF_1 and uncoupled electron transport and at higher concentrations either uncoupling is prevented or CF_1 may be rebound to the CF_1-depleted membrane.

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