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Anion (and Cation) Requirements of the Coupled Electron Flow in Spinach Thylakoids

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Abstract

Proton uptake as well as coupled electron flow of chloroplasts swollen in dilute impermeable buffers became dependent upon the addition of exogenous permeable anions. This dependence was observed with both cyclic and noncyclic electron acceptors, suggesting that this anion requirement is associated with the electrogenic proton uptake step rather than with the oxygen-evolving reactions of photosystem II.

Key Words: Light-induced electron flow; rate; coupled cations; anions; protons.

Introduction

Warburg and Lüttgens reported in 1944 that Cl^- was required for maximum rates of electron transport in chloroplasts. This Cl^- requirement was further investigated and assigned to the oxidizing side of photosystems II by Hind and his collaborators (Hind *et al.*, 1969; Heath and Hind, 1969a, Izawa *et al.*, 1969; Heath and Hind, 1969b; Kelley and Izawa, 1978). These workers noted, however, that Cl^- dependence of electron flow was often demonstrable only after the thylakoids had been heated at 40°C or exposed for brief periods to an alkaline pH. The effect of Cl^- on the rates of uncoupled electron transport were of primary interest in these experiments.

More recently, a dramatic dependence of O_2 evolution on Cl^- was demonstrated in chloroplasts from halophytic mangroves (Critchley *et al.*, 1982). In the thylakoids of some of these species, the anion specificity was very pronounced such that neither Br^- nor I^- substituted for Cl^-, in contrast

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to other species where I^- and to some extent NO_3^- anions were effective promoters of O_2 evolution. In spinach thylakoids the anion requirements, for example, are very broad (Critchley *et al.*, 1982) as sulfate, NO_3^-, Cl^-, Br^-, and I^- replace one another in the O_2 evolving reaction.

Recent reports, moreover, indicate that the anions in these species bind at more than one site. The $^{36}Cl^-$-NMR spectra suggested two sites for Cl^- binding in the stacked spinach thylakoid regions (Critchley *et al.*, 1982). Sinclair (1984) came to a similar conclusion from studies of Cl^- replacement effects on O_2 evolution with nitrate and sulfate. He noted that when nitrate replaced Cl^-, it decreased the rate constant of the reaction ($S_3 > S_0$) which limits the rate of electron flow on the oxidizing side of photosystem II. Sulfate, however, did not affect the rate constant of this reaction, but nevertheless inhibited electron flow.

In addition to the anion requirement in photosystem II, coupled electron flow is also associated with obligatory anion (or cation) fluxes. In 1972, Cohen and Jagendorf, reported that thylakoids swollen in an impermeable anion medium, sodium polygalacturonate, retained nearly normal electron flow, but lost more than 90% of proton uptake activity. The proton uptake activity of their polygalacturonate-swollen thylakoids could be restored by the addition of either NaCl, Na_2SO_4, or KSCN. These results indicate that the site(s) of the anion requirement associated with the proton uptake differs from the site of the anion requirement associated with the S states of photosystem II.

In the current study, we report that both proton uptake and coupled electron flow of thylakoids swollen in dilute, relatively membrane-impermeable buffers become dependent on added permeable anions. This dependence was observed with both cyclic and noncyclic electron acceptors, suggesting that this anion requirement is associated with the electrogenic proton uptake step rather than with the oxygen-evolving reactions of photosystem II.

Materials and Methods

Chloroplasts were isolated from 50 g of deveined spinach leaves and washed in a medium containing 0.4 M sucrose, 10 mM NaCl, and 50 mM Tricine,[3] pH 7.8, as previously described (Vambutas *et al.*, 1975). After one wash, each pellet was suspended in about 40 ml of cold 10 mM Hepes buffer adjusted to pH 7.7 with choline base. Prior to sedimentation at 2°C each

[3] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; Tricine, *N*-[2-hydroxyl-1,1-bis-(hydroxymethyl)ethyl]glycine; DCMU (diuron), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tris, tris(hydroxymethyl)aminomethane; PMS, phenazine methosulfate.

suspension was allowed to sit in ice (2–3°C) for a minimum of 5 min. The membranes were sedimented at 10,000 × *g* for 10 min and the pellets were suspended in cold 10 mM choline-Hepes buffer, pH 7.7, at 0.5–1.0 mg chlorophyll/ml shocked chloroplasts. Assays for proton uptake or O_2 evolution were performed 1–4 h after preparation. Nonshocked chloroplasts were prepared by the same procedure, except 10 mM choline-Hepes buffer, pH 7.7, used for swelling was supplemented with 0.2 M sucrose. After centrifugation, these chloroplasts were suspended in 0.2 M sucrose containing 5 mM choline-Hepes buffer, pH 7.7.

Chlorophyll concentration of the preparations was determined by the method of Arnon (1949). O_2 evolution or uptake was measured as described earlier (Cohn *et al.*, 1975) with $K_3Fe(CN)_6$ or methylviologen as the electron acceptors. NaN_3 was omitted from assays with methylviologen in order to control permeant ion presence. Only initial O_2 uptake rates were, therefore, recorded with methylviologen. Light-dependent proton uptake was measured in the presence of 2 mM Hepes buffer adjusted to pH 7–7.4 either with choline base or with Tris base (Cohen and Bertsch, 1974). Light-dependent proton uptake was standardized in the dark by addition of known amounts of standard HCl. Duroquinone prior to reduction was purified as follows: a beaker containing duroquinone was heated with a low flame till the duroquinone sublimed on the bottom of the watch glass covering the beaker. A 3-ml amount of purified duroquinone (25 mM in ethanol at about 5°C) was reduced by addition of 150 μl of 0.5 M KBH_4. A few minutes after KBH_4 addition, HCl was added to a final concentration of 8 μmol/ml. On acidification the solution became colorless and remained colorless for at least a few hours. This method for duroquinone purification and reduction was generously communicated by Dr. R. Dilley.

Materials. Gramicidin S, valinomycin, methylviologen, duroquinone (tetramethyl-*p*-benzoquinone), choline base, and choline chloride were purchased from Sigma; spinach was purchased from local markets.

Results

No light-dependent proton uptake was observed in chloroplasts that had been swollen in dilute Hepes buffers which had been adjusted to experimental pHs with choline base (Table I). Choline and Hepes were chosen because of their relative impermeability to the thylakoid membranes. Small amounts of proton uptake by shocked chloroplasts could not be measured accurately in the absence of KCl because of the electrode noise. The electrode response to molar amounts of HCl, however, under these conditions was in the normal range.

Proton uptake activity of swollen thylakoids reappeared on addition of

Table I. Restoration of Light-Dependent H^+ Uptake in Shocked Chloroplasts by Thylakoid Permeable Ions[a]

	Light-dependent H^+ uptake (nmol/mg chlorophyll)				
	Light-on: 1st	2nd	3rd	4th	5th
Experiment 1 (methylviologen)					
2 mM choline-Hepes, pH 7.3	< 10				
2 mM choline-Hepes, pH 7.3, + 20 mM KCl	231	241			
+ 2 μM DCMU			< 10		
2 mM K^+-Hepes, pH 7.54,	159	127	< 10		
+ 20 mM KCl				259	270
Experiment 2 (PMS)					
2 mM choline-Hepes, pH 7.22,	< 29	< 29			
+ 20 mM KCl			136	123	
+ 2 mM NH_4Cl					< 10
2 mM choline-Hepes, pH 7.44, + 20 mM KCl	326				
+ HCl, pH 7.08		342			
2 mM choline-Hepes, pH 7.48, + 20 mM KCl + 2 μM DCMU	90	135			
2 mM K^+-Hepes, pH 7.3,	186	< 10			
+ 20 mM KCl			226	266	

[a]Chloroplasts were shocked in 10 mM choline-Hepes, pH 7.7, as described in Materials and Methods. The same preparation of shocked chloroplasts was used in experiments 1 and 2. Each reaction mixture contained in a final volume of 5 ml: 0.1 mM methylviologen or 0.05 mM PMS; shocked chloroplasts, 34.6 μg chlorophyll/ml; and other additions as indicated in the table.

KCl (permeant ions) to the assay medium (Table I). This restored proton uptake activity was completely abolished by DCMU with methylviologen as the electron acceptor. Addition of the thylakoid-permeant cation, K^+, in the absence of the permeant anion, Cl^-, sustained only a temporary proton uptake which ceased after the first or second light-on. Potassium chloride added to this system after the third light-on, after the proton uptake had ceased, once again restored proton uptake (Table I).

Similarly, no light-dependent proton uptake by swollen thylakoids was observed in the presence of impermeable ions and a cyclic electron donor/acceptor, PMS (Table I, experiment 2). Potassium chloride added after the second light-on restored proton uptake activity which was subsequently abolished by an added uncoupler, NH_4Cl. The cyclic nature of the electron flow can be inferred from the lack of substantial inhibition of proton uptake by DCMU. Higher proton uptake activities were obtained if KCl was added before the first light-on rather than after the first or second, suggesting that the absence of permeant ions during illumination may have resulted in some irreversible damage to the thylakoids. With PMS, as with methylviologen, proton uptake activity ceased in a medium containing only permeant cations (K^+). This activity reappeared upon addition of KCl (Table I, experiment 2).

Table II. Anion Species vs Extent of Light-Dependent H^+ Uptake by Shocked Chloroplasts[a]

	H^+ uptake (nmol/mg chlorophyll)				Activity (%)
	Light-on: 1st	2nd	3rd	4th	
Experiment 1 (methylviologen)					
No additions	< 10				
+ 20 mM KCl		200	211		
+ 20 mM KCl	370				100
+ 20 mM KNO_3	324	297			88
+ 20 mMKI	282	245			76
+ 20 mM K_2SO_4	203	216			55
+ 20 mM KCl			230	203	
Experiment 2 (methylviologen)					
No additions	182	154			33
+ 10 mM KCl	553	523			100
+ 10 mM choline-SO_4	224	194			41
+ 10 mM choline-Cl	538	523			97
Experiment 3 (PMS)					
No additions	164				26
+ 10 mM KCl	627	597	523		100
+ 10 mM choline-Cl	523	523	523		83
+ 20 mM choline-SO_4	209	239			33
+ 10 mM NaSCN	553	597			88
+ 10 mM KCl + 2 μM DCMU	299	164			48

[a]The reaction mixtures contained in a final volume of 5.0 ml: 2 mM Tris-Hepes buffer, pH 7.2, 0.1 mM methylviologen or 0.05 mM PMS, 37 μg of chlorophyll in experiments 1 and 44 μg/ml of reaction mixture in experiments 2 and 3. Thylakoids for experiment 1 were swollen in 10 mM Tris-Hepes, pH 7.2; for experiments 2 and 3 in 10 mM choline-Hepes, pH 7.7. Other additions to the reaction mixtures were as indicated in the table. The pHs of the reaction mixtures ranged from 6.85 to 7.06.

Since Cl^- seemed to play a more important role in restoring proton uptake in choline-Hepes swollen thylakoids, anion specificity was explored in the following experiment. Thylakoids swollen in Tris-Hepes were assayed for light-dependent proton uptake activity in Tris-Hepes medium with methylviologen as the electron acceptor. In these experiments, Hepes was neutralized with Tris base to pH 7.1. At this pH the primary amino group of Tris base should be in the protonated, membrane-impermeable state. The results summarized in Table II, experiment 1 demonstrate again a lack of proton uptake in the absence of permeant ions, and restored proton uptake activity upon addition of KCl after the first light-on to thylakoids swollen in Tris-Hepes buffer. If restoration of proton uptake by 20 mM KCl added before illumination is assumed to be 100%, then the equivalent concentration of KNO_3 (Table II, experiment 1) restored proton uptake to 88%; KI, to 76%; and K_2SO_4, to 55%. KCl added after the second light-on, after the initial addition of K_2SO_4, failed to increase proton uptake. Of the permeable anions (in the absence of permeable cations) Cl^- was the most effective in restoring

proton uptake (Table II, experiment 2). Interesting, sulfate in the absence of permeable cations had almost no activity, while sodium thiocyanate, a lipid-permeable anion, was as effective as KCl.

Two opposite interpretations of the observed results are immediately apparent: (1) absence of permeant ions induces an uncoupled state of electron flow and/or (2) electrons do not flow through a coupled electron transport chain in the absence of permeant ions. Electron transport was, therefore, measured under conditions similar to those employed in proton uptake measurements. Experiments summarized in Table III, experiment 1, part (a) show low electron flow activity with $K_3Fe(CN)_6$ as an electron acceptor in choline-Hepes shocked chloroplast, in the absence of permeant anions or cations. The rate of coupled electron flow was increased almost fourfold upon addition of either KCl, choline-Cl, or K^+-Hepes, pH 7.7. Gramicidin increased the rate of electron flow nearly threefold in reactions which were supplemented with KCl or with choline-Cl, indicating the existence of significant proton pools within these thylakoids. Gramicidin, however, did not enhance the rate of electron flow with K^+ alone in the medium. Not only did gramicidin fail to enhance the rate of electron flow, but it also caused a significant decrease (Fig. 1). The results obtained with methylviologen as the electron acceptor corraborated the observations made with $K_3Fe(CN)_6$ (Table III, experiment 2). Gramicidin again failed to enhance the rate of electron flow in K^+-Hepes medium. The actual O_2 evolution rates (traces) are shown in Fig. 1. Choline-Cl clearly stimulates the rate of O_2 evolution in shocked chloroplasts and this rate is further enhanced by gramicidin. Potassium Hepes has no effect and valinomycin or gramicidin added together with K^+ decreases the residual rate of O_2 evolution.

Very different results were obtained if the thylakoid wash and assay medium contained sucrose in addition to choline-Hepes [Table III, experiment 1, part (b)]. The size of the thylakoid pellet indicated that these thylakoids were less swollen than the thylakoids washed in choline-Hepes medium in the absence of sucrose. It is possible that thylakoids swollen in choline-Hepes had lost internal inorganic ions, whereas thylakoids washed, suspended, and assayed in a medium containing sucrose retained a sufficient amount of ions (cations) necessary for coupled electron flow in the absence of added permeable ions. Under these conditions (absence of permeable ions in the assay medium) the electron flow rates of unshocked chloroplasts were partially uncoupled [Table III, experiment 1, part (b)]. The uncoupled condition disappeared when the permeable ions were included in the assay medium. Gramicidin enhanced the rate of electron flow to about the same extent in any of the four assay buffers [Table III, experiment 1, part (b)] and to about the same extent as in an electron-flow restored system [Table III, experiment 1, part (a)]. It should be noted that the swollen and the

Table III. Restoration of Coupled Electron Flow in Shocked and Nonshocked Chloroplasts by Thylakoid Permeable Ions[a]

Shocked chloroplasts	O_2 evolution (μmol/h/mg chlorophyll)		Nonshocked chloroplasts	O_2 evolution (μmol/h/mg chlorophyll)	
Experiment 1, part (a) ($K_3Fe(CN)_6$)	–	+ Gramicidin	Experiment 1, part (b) ($K_3Fe(CN)_6$)	–	+ Gramicidin
No additions	22	23	No additions	136	229
+ 22 mM KCl	82	228	+ 22 mM KCl	32	245
+ 22 mM choline-Cl	82	255	+ 22 mM choline-Cl	43	266
+ 22 mM K^+-Hepes, pH 7.7	80	31	+ 22 mM K^+-Hepes, pH 7.7	58	208
	O_2 consumption (μmol/h/mg chlorophyll)			O_2 consumption (μmol/h/mg chlorophyll)	
Experiment 2 (methylviologen)	–	+ gramicidin	Experiment 3 (methylviologen)		
No additions	41	–	No additions	114	
+ 22 mM KCl	85	123	50 mM choline-Hepes, pH 7.7	100	
+ 22 mM choline-Cl	68	108	10 mM KCl	23	
+ 22 mM K^+-Hepes, pH 7.7	37	34	50 mM KCl	59	
			10 mM choline-Cl	63	
			50 mM choline-Cl	61	
			10 mM K^+-Hepes, pH 7.7	65	
			50 mM K^+-Hepes, pH 7.7	61	

[a] Chloroplasts were shocked in 10 mM choline-Hepes, pH 7.7, as described in Materials and Methods for experiment 1, part (a) and for experiment 2. For experiment 1, part (b) and experiment 3, isolated chloroplasts were washed, suspended, and assayed in 0.2 M sucrose containing 5 mM choline-Hepes, pH 7.7. The assay medium in experiment 1, part (a) consisted of: 10 mM choline-Hepes buffer, pH 7.7; 0.4 mM $K_3Fe(CN)_6$; shocked chloroplasts, 23.9 μg chlorophyll/ml; and other additions as indicated in the table in a total volume of 5 ml. Nonshocked chloroplasts assayed in experiment 1, part (b) were derived from the same batch of chloroplasts as the shocked chloroplasts in experiment 1, part (a). The assay medium in experiment 1, part (b) contained: 0.2-M sucrose; 5 mM choline-Hepes buffer, pH 7.7; 0.4 mM $K_3Fe(CN)_6$; and unshocked chloroplasts, 20.5 μg/chlorophyll/ml of reaction mixture. In experiment 2, $K_3Fe(CN)_6$ was replaced with 0.10 mM methylviologen and O_2 uptake was measured with shocked chloroplasts, 18.8 μg/chlorophyll/ml of reaction mixture in the absence of sucrose and with other additions as indicated in the table. In experiment 3, unshocked chloroplasts were assayed for O_2 uptake in the presence of: 0.2 M sucrose; 5 mM choline-Hepes, pH 7.7; 0.1 mM methylviologen; unshocked chloroplasts, 45.5 μg/chlorophyll/ml; and other additions as indicated in the table. The conditions for measuring light-dependent O_2 evolution or uptake were as described by Cohen and Bertsch (1974).

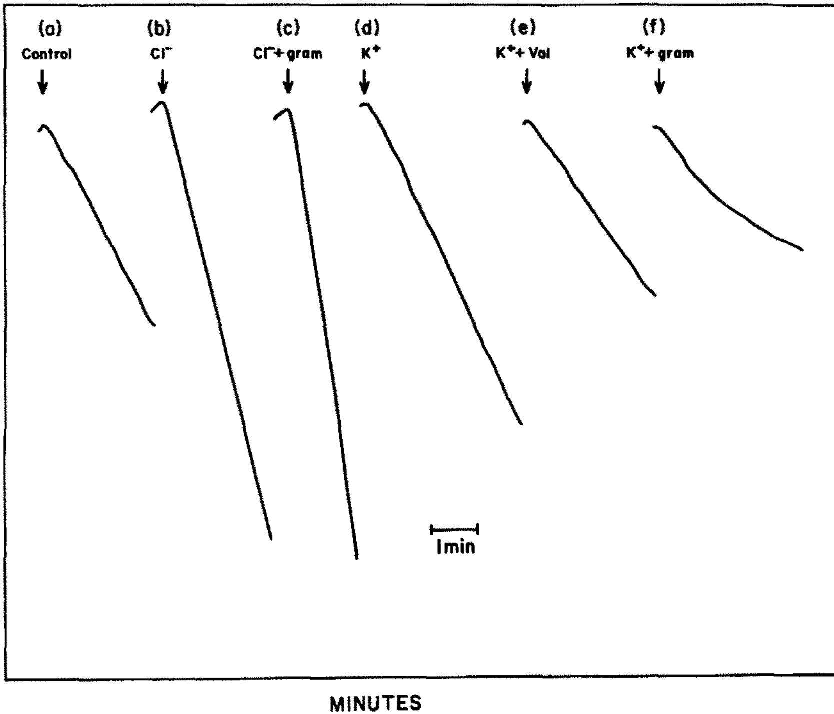

Fig. 1. Differential K^+ and Cl^- effects on O_2 evolution of shocked chloroplasts. Reaction conditions were as described in Table V for twice shocked chloroplasts. Each reaction mixture contained 5 mM choline-Hepes, pH 7.7, 0.4 mM $K_3Fe(CN)_6$, and 35.7 μg/ml chlorophyll/ml; (a) no additions; (b) 10 mM choline-Cl; (c) 10 mM choline-Cl + 1 μM gramicidin; (d) 10 mM K^+-Hepes buffer, pH 7.8; (e) 10 mM K^+-Hepes buffer, pH 7.8, +2.2 μg/ml valinomycin; (f) 10 mM K^+-Hepes buffer, pH 7.8, + 1 μM gramicidin. Arrow indicates start of illumination.

nonswollen thylakoids in parts (a) and (b) were derived from the same batch of chloroplast preparation and, hence, a valid comparison can be made.

These results suggested that the lack of proton uptake by shocked chloroplasts resulted from a lack of electron flow. The lack of electron flow might have resulted from a deprivation of the water oxidizing reaction associated with PSII of Cl^-. To test this hypothesis, durohydroquinone which, according to Izawa and Pan (1978), donates electrons in a DBMIB-sensitive reaction to plastoquinone, was employed to bypass PSII. The results summarized in Table IV show that Cl^- and to a lesser extent K^+ stimulated both the coupled and the uncoupled electron flow from durohydroquinone to methylviologen under conditions where the electron flow from PSII was completely blocked by DCMU. Increased dependence of electron flow on added ions (Table IV, experiment II) in this experiment appears to

Table IV. Restoration of Electron Flow from Durohydroquinone to Methylviologen in Shocked Chloroplasts by Thylakoid Permeable Ions

	O_2 uptake (μmol/h/mg chlorophyll)	
	Experiment I	Experiment II
Part (a): $H_2O \rightarrow$ methylviologen		
No additions	25	
$+1\,\mu$M gramicidin	39	
+20 mM choline-Cl	59	
+20 mM choline-Cl + $1\,\mu$M gramicidin	133	
+20 mM choline-Cl + $4\,\mu$M DCMU	0	
Part (b): Duroquinol (+DCMU) $\rightarrow$ methylviologen		
No additions	162	29
+20 mM choline-Cl	254	165
+20 mM KCl	272	136
+20 mM K^+-Hepes, pH 7.7	212	113
$+1\,\mu$M gramicidin	471	81
+20 mM choline-Cl + μM gramicidin	711	567
+20 mM KCl + μM gramicidin	646	485
+20 mM K^+-Hepes, pH 7.7, + μM gramicidin	526	386

[a]The reaction mixtures in experiment I, parts (a) and (b) contained in a final volume of 5 ml: 10 mM choline-Hepes buffer, pH 7.7; 0.1 mM methylviologen; and 17.9 μg chlorophyll/ml of reaction mixture of thylakoids swollen in 10 mM choline-Hepses, pH 7.7. The reaction mixtures shown in part (b) contained also 4 μM DCMU and 0.38 mM of durohydroquinone. The reaction mixtures in experiment II were as described in experiment I except 10 mM choline-Hepes buffer was replaced with 2 mM choline-Hepes, pH 7.7, and chloroplasts were swollen in 3 mM choline-Hepes buffer, pH 7.3, instead of 10 mM. Chlorophyll concentrations was 18.8 μg/ml of reaction mixture.

correspond to decreased concentration (from 10 to 3 mM) of choline-Hepes, chloroplast wash, and assay buffer. The K^+ (0.38 mM) and Cl^- (0.16 mM) added with the durohydroquinone to the reaction mixtures may or may not have been responsible for the gramicidin enhancement of the residual electron flow in the absence of "extra" Cl^- or K^+. The electron flow from durohydroquinone to methylviologen was also coupled to H^+ uptake in the presence, but not in the absence, of added Cl^-. For example, at pH 7.6, 322 nmol of H^+/mg chlorophyll dissappeared from the reaction mixture containing 20 mM choline-Cl, whereas no change in H^+ concentration was detected in reaction mixtures lacking choline-Cl (Table IV, experiment II). The O_2 consumption rates with the durohydroquinone–methylviologen couple were probably inflated due to the oxidation of durohydroquinone by $O_2^{\cdot -}$, which is the initial product of O_2 reduction by reduced methylviologen (Izawa and Pan, 1978). These observations taken together with the observed anion requirement for proton uptake with a cyclic electron acceptor, PMS, seemed to suggest that the electron flow was controlled by ions through its coupling mechanism.

Table V. Potassium Stimulation of Electron Flow in Shocked Chloroplasts and Removal of This Activity by Additional Shocking[a]

	O_2 evolution (μmol/h/mg chlorophyll) after		Activity decrease (%)
	1st shock	2nd shock	
No additions	16.0	16.3	0
+10 mM choline-Cl	38.6	34.6	10
+10 mM choline-Cl + 1 μM gramicidin	–	54.3	
+10 mM K^+-Hepes, pH 7.8	25.4	17.3	32
+10 mM K^+-Hepes, pH 7.8 + 1 μM gramicidin	65.9	10.9	83
+10 mM K^+-Hepes, pH 7.8	24.5	–	
+10 mM K^+-Hepes, pH 7.8 + 2.2 μg/ml valinomycin	24.5	10.9	55
+10 mM K^+-Hepes, pH 7.8, +10 mM choline-Cl	33.9	32.6	4
+10 mM K^+-Hepes, pH 7.8, +10 mM choline-Cl + 1 μM gramicidin	92.3	69.2	25
+10 mM K^+-Hepes, pH 7.8, +10 mM choline-Cl	33.9		
+10 mM K^+-Hepes, pH 7.8, +10 mM choline-Cl + 2.2 μg/ml valinomycin	33.9		
+10 mM choline-SO_4	35.8	13.3	63
+10 mM choline-SO_4 + 1 μM gramicidin	52.8	8.9	83

[a]The reaction mixture contained in a final volume of 5 ml: 5 mM choline-Hepes, pH 7.7, 0.4 mM $K_3Fe(CN)_6$, 37.4 μg chlorophyll for once shocked chloroplasts and 35.7 μg/ml for twice shocked chloroplasts. The first swelling was performed as described in Materials and Methods. Since this batch of chloroplasts retained K^+ stimulating activity of electron transport, these chloroplasts were subjected to a second swelling in cold 5 mM choline-Hepes buffer, pH 7.7, at chlorophyll concentrations of about 0.3 mg/ml. Potassium concentration in 10 mM Hepes buffer was 6 mM. Proton uptake with two times shocked chloroplasts was 280 nmol/mg chlorophyll in the presence of PMS and 10 mM KCl.

Some variation was observed in shocked chloroplast preparations. Occasionally, K^+ alone restored coupled electron flow, whereas in others this activity was lacking. The results summarized in Table V show that an additional washing of swollen thylakoids that were stimulated by K^+, in dilute choline-Hepes buffers, removed this activity (and the sulfate anion activity as well). How the added K^+ entered the thylakoid is unclear—possibly in exchange with some other ion that had not been removed. In any event, the K^+ pathway ceased to function after the second swelling, though the electrogenic Cl^- pathway remained operational.

Discussion

The results of this study show that chloroplasts shocked in the absence of permeable anions and cations in the medium lost 70–100% of their proton uptake activity and underwent a similar decrease in the uncoupled electron transport rate. Cohen and Jagendorf (1972) similarly observed more than a decade ago that chloroplasts shocked (swollen) in the presence of permeant cations and impermeant anions lost proton uptake activity, which they were able to restore by readdition of NaCl. Electron flow, however, was not impaired under their experimental conditions. Permeant cations which were present in their chloroplast swelling media can perhaps account for the differences between their observations and ours.

What could be the effect of the presence of permeant cations in the swelling medium?

Cation concentrations within the thylakoids might remain high. Upon illumination, the efflux of these cations might be necessary to neutralize the membrane potential arising from either the initial separation of charges or from the electrogenic proton influx, thus permitting optimal rates of electron flow. Indeed, we find that residual electron flow in choline-Hepes swollen thylakoids is further inhibited by K^+-Hepes–gramicidin couple or K^+-Hepes–valinomycin couple. (In each case a positive diffusion potential was directed inward, Fig. 1).

Proton gradient effects on electron flow rates are well known. Fully uncoupled rates where no H^+ gradient across the membrane is established are usually several times greater than coupled rates. Phosphorylating conditions enhance the rate of electron flow above the basal rate by enhancing the efflux of H^+. Added ADP or ATP (which prevent H^+ leakage through CF_1) reduce electron flow rates by 30–40% below the basal flow (Vambutas and Bertsch, 1975). Inhibition of electron flow rates by cations other than H^+ has also been observed (Gross *et al.*, 1969).

Higher rates of electron flow were also observed upon omission of permeant ions from the assay medium of normal, unshocked chloroplasts

(broken, but not swollen, and therefore still possessing some soluble proteins and ions) (Table III). Such enhanced rate might suggest partial uncoupling —leakage of protons from the thylakoids into the medium. Added permeant anions or cations significantly reduced the rate of electron flow in these chloroplasts (Table III). Gross *et al.* (1969) similarly noticed "recoupling" of sucrose-uncoupled thylakoids by added cations.

Whereof comes the difference between shocked and unshocked chloroplasts? Shocked chloroplasts were probably depleted of both permeable anions and cations in contrast to the unshocked chloroplasts which probably had a reasonable store of both. If counter-ion exchange (K^+) or co-ion (Cl^-) uptake could not occur, then an electrogenic proton uptake would develop a large electrical potential which could stop further proton uptake. It has been calculated that 1 H^+ per chlorophyll could generate a potential of about 300 mV if the charges were not neutralized (Dilley, 1971). Inability of uncouplers to release this inhibition (uncouplers should also destroy $\Delta\psi$ due to H^+ uptake) in the absence of anions suggests perhaps a catalytic anion requirement for this reaction. The coupled electron flow could be affected, for instance, through the plastoquinone site—pile-up of reduced species.

Indeed, the rate of durohydroquinone oxidation in swollen thylakoids when the electron flow from PSII is blocked by DCMU is very substantially stimulated by added anions (or cations) (Table IV). This observation places the ion requirement(s) of the electron flow in swollen thylakoids at or past the plastoquinone site. A somewhat more pronounced anion (or cation) requirement was observed with H_2O as the electron donor than with durohydroquinone (Table IV). At present it is not known to what extent, if any, PSII in the swollen thylakoids was depleted of Cl^-. A slight indication was obtained in the experiments with uncouplers. Although the coupled rate is restored by addition of K^+ [Table III, part (a)] the uncoupled rate is not, but indeed decreases severely with time. The uncoupler in this experiment, in accordance with an earlier report (Theg and Homann, 1982), may have rapidly depleted PSII of Cl^-. If this interpretation is correct, PSII of swollen thylakoids is not depleted of Cl^-.

The anion specificity of the second anion-requiring site is unknown. The results shown here might reflect the specificity of a transporter plus the specificity of the second yet poorly understood reaction of the coupled (uncoupled) electron flow.

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