Evidence for Ca^{2+}-Gated Proton Fluxes in Chloroplast Thylakoid Membranes: Ca^{2+} Controls a Localized to Delocalized Proton Gradient Switch†

Gisela Chiang and Richard A. Dilley*
Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907
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ABSTRACT: Chloroplast thylakoid ATP formation can be driven by proton gradients, which are experimentally detected as either delocalized or localized by the accepted criteria of the ATP formation onset lag (the need to reach the energetic threshold sufficient to drive ATP formation) and postillumination phosphorylation sensitivity to added permeable buffers such as pyridine. Thylakoids stored in a low-salt, 200 mM sucrose containing buffer were insensitive to pyridine (a localized proton gradient response), whereas storage in a 100 mM KCl containing medium resulted in sensitivity of both phosphorylation parameters to pyridine, showing delocalized H^+ gradient behavior. The KCl effect seems to be mediated by divalent cations, particularly Ca^{2+}, as indicated by the following: (A) 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), a Ca^{2+} chelator added to the low-salt, sucrose-containing medium, was nearly as effective as 100 mM KCl in causing the pyridine-induced effects on the phosphorylation parameters; (B) 1 mM $CaCl_2$ or 30 mM $MgCl_2$, added to the 100 mM KCl containing medium, blocked the action of the 100 mM KCl medium, maintaining the thylakoids in a localized coupling response; (C) when 2 mM EGTA was also present with either the 30 mM $MgCl_2$ or the 1 mM $CaCl_2$ medium, it blocked the divalent cation tendency to promote the localized coupling response, resulting in a return to a delocalized coupling response; and (D) a membrane-permeable Ca^{2+} chelator, added to the low-salt thylakoid storage medium, caused the normally localized behavior of those thylakoids to be switched to a delocalized coupling mode. The results suggest that Ca^{2+} ions associated with the lumenal side of thylakoid membranes or with the interior of the membrane in some way regulate a gating function that can switch proton diffusion pathways from localized to delocalized energy coupling gradients.

Recent experiments have resolved a long-standing controversy concerning whether chloroplast thylakoid proton gradients that drive ATP formation are solely delocalized, a position held by some researchers (Davenport & McCarty, 1980; Vinkler et al., 1980), or can be localized in some cases (Ort et al., 1976; Graan et al., 1981). Delocalized gradient coupling is readily demonstrated by the effects of permeable buffers on acid–base phosphorylation (Uribe & Jagendorf, 1968) or postillumination ATP formation (Nelson et al., 1971). There is no disagreement with the interpretation that protons associated with permeable buffers, present in thylakoid lumen, can increase the yield of acid–base driven or postillumination phosphorylation (PIP).[1] The controversy, referred to above, arose in applying the permeable buffer approach to measuring the length of the ATP formation energization lag, a parameter that should be predictably correlated with increases in the lag length when delocalized coupling occurs or with no effect on the lag length if the proton gradient is localized. Some results clearly showed that buffers such as pyridine (Vinkler et al., 1980) or imidazole (Davenport & McCarty, 1980) delayed the onset of energization as expected for the delocalized coupling model, but other results just as clearly showed no effect of several buffers, including pyridine (Ort et al., 1976; Graan et al., 1981; Horner & Moudrianakis, 1983, 1986), on the energization lag, consistent with a localized proton gradient coupling mechanism.

The resolution of the controversy is that both data sets are valid, inasmuch as recent results show that conditions such as salt concentration used to store thylakoids determine whether the proton gradient driving ATP formation does or does not interact with an added permeable buffer. Beard and Dilley (1986) [cf. also Horner and Moudrianakis (1983)] showed that storing thylakoids with a low-salt, high-sucrose medium resulted in ATP formation energization lags (and postillumination phosphorylation) not sensitive to pyridine, in agreement with earlier results (Ort et al., 1976; Graan et al., 1981), but when aliquots of the same thylakoid preparation were stored in media with 100 mM KCl, pyridine effects on the energization lag were clearly observed. The lack of a pyridine effect on the energization lag reported in the earlier work was the controversial part, as mentioned above. The lack of a pyridine effect on the postillumination ATP yield was a new finding in the recent work of Beard and Dilley (1986, 1987a) and Beard et al. (1987) [Beard et al. (1987) note a new way of assaying postillumination phosphorylation], and it greatly strengthens the hypothesis that in the low-salt-treated thylakoids a strictly localized, nonlumenal pathway exists for the buildup and utilization of the proton gradient in the energy-coupling mechanism.

An important finding, and one lending more credibility to the controversial, localized gradient hypothesis, was that the same thylakoid material, stored with 100 mM KCl in place of 200 mM sucrose, resulted in clearly measurable and predictable pyridine effects (based on the delocalized gradient theory) on both the ATP onset lag and on postillumination

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[1] Abbreviations: PIP, postillumination phosphorylation; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Chl, chlorophyll; ATP, adenosine 5′-triphosphate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DTT, dithiothreitol; P_i, inorganic phosphate; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(dimethylamino)octyl ester; PSI and PSII, photosystems I and II; CF_1 and CF_o, coupling factors 1 and o.

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Table I: Effect of Salts and EGTA on Phosphorylation Parameters[a]

treatment	energization lag, no. of flashes		lag difference due to pyridine, (+Pyr) − (−Pyr)	PIP^+ ATP yield [nmol of ATP (mg of Chl)$^{-1}$]	
	−Pyr	+Pyr		−Pyr	+Pyr
(1) control	29/37[b]	30/40	1/3	2.8 ± 0.4	3.0 ± 0.5
(2) 100 mM KCl	37/52	52/71	15/19	3.3 ± 0.2	6.7 ± 0.2
(3) 100 mM KCl, 1.0 mM $CaCl_2$	39/52	42/60	3/8	3.4 ± 0.1	4.7 ± 0.1
(4) 100 mM KCl, 30 mM $MgCl_2$	32/47	35/53	3/6	2.4 ± 0.2	2.6 ± 0.2
(5) 100 mM KCl, 30 mM $MgCl_2$, 2 mM EGTA	31/45	46/63	15/18	3.7 ± 0.3	7.8 ± 0.7
(6) 100 mM KCl, 1 mM $CaCl_2$, 2 mM EGTA	37/54	49/71	12/17	4.1 ± 0.2	7.0 ± 0.5
(7) 100 mM KCl, 30 mM $MgCl_2$, 2 mM EGTA, 4 mM $CaCl_2$	41/54	43/62	2/8	3.6 ± 0.2	5.8 ± 0.5

[a] Thylakoids were resuspended after isolation in either the low-salt medium (control), the 100 mM KCl containing medium (high salt) as defined under Materials and Methods and in the legend of Figure 1, or medium with the addition of the various $CaCl_2$, $MgCl_2$, and EGTA combinations listed for treatments 3–7. The storage conditions and the subsequent dilution into the ATP formation assay medium are given under Materials and Methods. Where present, pyridine was added at 5 mM 3.5 min prior to starting the 5-Hz flash sequence. The two lag parameters are defined briefly in Figure 1, and in more detail in Beard and Dilley (1986), and are the average of at least three independent assays. The PIP^+ ATP yield refers to postillumination phosphorylation that occurred after the last flash of a sequence during which ATP was formed (ADP and P_i were present from the beginning of the flash sequence). [b] Average of three or more assays. Standard deviations were ±1 or ±2 in all cases except line 2 (−Pyr), where the data were 52 ± 3.

phosphorylation (Beard & Dilley, 1986, 1987b). Detailed studies (Beard & Dilley, 1987b) failed to reveal any trivial artifacts to explain the data but rather gave a broad base supporting the hypothesis that storing thylakoids in 100 mM KCl buffer medium in some way causes a type of gating [cf. also Horner and Moudrianakis (1986)] so as to allow protons, which in the low-salt treatment case appear to be constrained to a nonlumenal pathway (i.e., a localized proton gradient), to be shunted to the lumen where they interact predictably with the added permeable buffers, in accord with the delocalized gradient-coupling concept.

To test further the validity of the KCl-induced proton gradient gating hypothesis and to clarify the mechanism of the apparent switching effect of the KCl treatment, we carried out experiments to learn what factors are involved in the phenomenon. This paper will show that KCl and other ion effects, when either localized or delocalized coupling responses are observed, seem to be mediated by Ca^{2+} ions present in the thylakoids.

Materials and Methods

Spinach thylakoids were prepared from growth chamber growth spinach as described before (Beard & Dilley, 1986), except that leaves were dark adapted at least 3 h before thylakoid isolation. The standard suspension medium for storing thylakoids after isolation for the low-salt medium was 200 mM sucrose, 5 mM Hepes–KOH (pH 7.5), 2 mM $MgCl_2$, and 0.5 mg/mL bovine serum albumin. In the high-salt medium, 200 mM sucrose was replaced by 30 mM sucrose and 100 mM KCl. Additional salt and/or EGTA were (was) included in either of those media, at concentrations specified in Table I or the figure legends. After being stored in the particular medium for 30 min or more at ice bath temperatures and at a chlorophyll concentration of about 3–4 mg mL^{-1}, aliquots were diluted to 15 μg of Chl mL^{-1} into the ATP formation assay medium consisting of 50 mM Tricine–KOH (pH 8.0), 10 mM sorbitol, 3 mM $MgCl_2$, 1 mM K_2HPO_4, 0.1 mM ADP, 5 mM DTT, 400 nM valinomycin, luciferin luciferase prepared from the LKB ATP assay kit as described in Beard and Dilley (1986), 5 μM diadenosine pentaphosphate (Sigma), and 0.1 mM methylviologen. The special assay cuvette, maintained at 10 °C, was illuminated by single-turnover flashes (5-Hz repetition rate) and the ATP-dependent luciferin luciferase luminescence was detected as described before (Beard & Dilley, 1986). The ATP formation energization lag was estimated by two parameters, the number of flashes required to elicit the first detectable rise in luminescence and the extrapolated lag number, as described in Beard and Dilley (1986).

Results

The gating of the proton gradient from a localized to a delocalized mode is indicated when a treatment induces a pyridine-dependent increase in the ATP formation onset flash lag number and pyridine-dependent effects on postillumination ATP yield (Beard & Dilley, 1986, 1987b). The localized gradient response is found in thylakoids stored in the standard low-salt medium, which typically showed ATP formation energization lags as in Figure 1A. Addition of 5 mM pyridine (the bottom curve in Figure 1A) gave little or no lag extension (Table I, line 1). Those results and the lack of pyridine effect on the postillumination ATP yield [termed PIP^+, Figure 1A and Table I; for details see Beard and Dilley (1986, 1987a)] are expected for localized proton gradient characteristics. It should be noted that the same low-salt-treated thylakoids were shown to give delocalized H^+ gradient responses to pyridine when the traditional postillumination phosphorylation protocol was performed (Beard & Dilley, 1987a; Beard et al., 1987), wherein the proton accumulation occurs in the absence of ADP and P_i and is of greater magnitude than the proton uptake taking place during coupling conditions. That delocalized gradient effect was interpreted as owing to the proton fluxes being gated into the lumen after the proton-buffering capacity of the membrane-phase localized domains was saturated (Beard et al., 1987; Beard & Dilley, 1987b). The point to be made here is that the phenomenon we refer to as a gating response to the delocalized mode can be observed in the low-salt-treated thylakoids (which in the usual assay show localized coupling) as well as demonstrated by comparing the responses of low-salt-treated and 100 mM KCl treated thylakoids, as shown below.

Delocalized proton gradient coupling is indicated in our assay when pyridine causes an extension of the ATP formation onset lag and, for pH 8 conditions, when pyridine increases the PIP^+ ATP yield (Beard & Dilley, 1986, 1987b). That effect, caused by storage in buffer containing 100 mM KCl, is shown in Figure 1B; pyridine increased the ATP onset lag parameters from 37/52 to 52/71 (Table I, line 2), and the PIP^+ ATP yield was increased from 3.3 to 6.7 nmol of ATP (mg of Chl)$^{-1}$. The justification for identifying the response to the KCl treatment as a gating phenomenon rather than a nondescript salt effect—such as washing away a peripheral

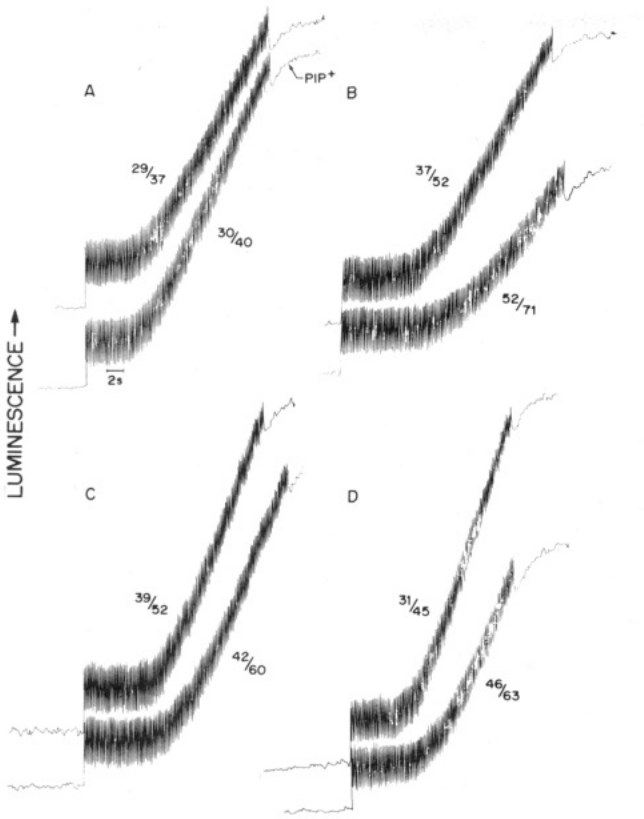

FIGURE 1: Effect of ionic composition and EGTA, with and without pyridine, on the energization lag for ATP formation and on postillumination phosphorylation. The thylakoid storage treatment, phosphorylation medium, and luciferin luciferase ATP assay were as described under Materials and Methods. The energization lag parameters are listed by each trace; i.e., for (A), top trace, the top number 29 is the number of flashes to the first detectable rise in the luminescence signal and the bottom number 37 is the flash number estimated by the intersection of the line given by the bottom of the rising signal and the extension of the horizontal line defined by the bottom of the flashes occurring during the onset lag. The spikes are actinic light leaks passed by the cutoff filter used to transmit the luminescence, as described in Beard and Dilley (1986, 1987a). In assays A–D, the top trace was from a sample without pyridine and the bottom trace was from a sample with 5 mM pyridine present, 3.5 min before beginning the flashes. Thylakoid samples were stored in the following media prior to dilution (approximately 5 μL added to 800 μL of reaction medium) into the phosphorylation medium (identical for all samples). (A) Low salt (control): 200 mM sucrose, 5 mM Hepes–KOH, pH 7.5, 2 mM $MgCl_2$, and 0.5 mg mL^{-1} bovine serum albumin. (B) High salt: 100 mM KCl and 30 mM sucrose replacing the 200 mM sucrose used in (A). (C) High salt + 1 mM $CaCl_2$: the storage medium as in (B) plus 1 mM $CaCl_2$. (D) High salt + 30 mM $MgCl_2$ + 2 mM EGTA: the medium as in (B) plus 30 mM $MgCl_2$ and 2 mM EGTA. In (A), the bottom trace identifies the postillumination phosphorylation ATP yield (PIP^+).

protein, causing an irreversible change in the energy-coupling responses—is, in part, that the effect was reversible upon washing the KCl-treated thylakoids with a low-salt, high-sucrose medium [Table 4 of Beard and Dilley (1987b)]. Furthermore, as shown below, the high-salt storage step is not absolutely required to observe the switching to the delocalized mode, and there appears to be specific Ca^{2+} effects that reverse the delocalizing effect of the KCl treatment.

The pyridine effect of extending, by about 15 flashes, the ATP formation onset lag in thylakoids stored in 100 mM KCl containing buffer was largely abolished when 1 mM $CaCl_2$ or 30 mM $MgCl_2$ was added to the storage buffer (Table I, lines 3 and 4; Figure 1C). Figure 2 shows the concentration curves for those $CaCl_2$ and $MgCl_2$ effects on the pyridine-dependent flash lag increase in the KCl-treated thylakoids.

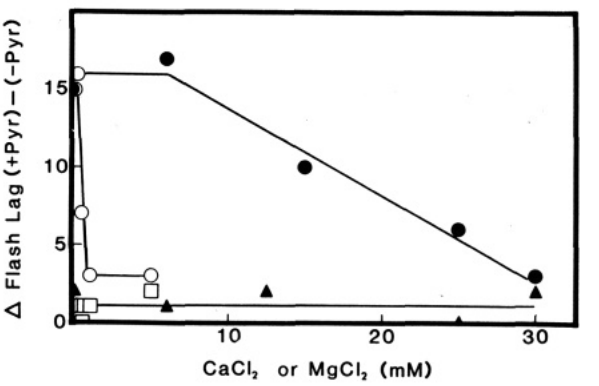

FIGURE 2: Effect of $CaCl_2$ and $MgCl_2$ added to the thylakoid storage buffer on the pyridine extension of the ATP formation onset lag. Chloroplasts were stored in either high-salt or low-salt resuspension medium as described under Materials and Methods with the addition of increasing concentrations of $CaCl_2$ or $MgCl_2$. The ATP onset lags were determined as described in Figure 1. The Δ flash lag was found by subtracting the flash lags in the absence of pyridine from that in the presence of pyridine. (●) High-salt thylakoid preparation, $MgCl_2$; (▲) low-salt thylakoid preparation, $MgCl_2$; (○) high-salt thylakoid preparation, $CaCl_2$; (□) low-salt thylakoid preparation, $CaCl_2$.

However, in thylakoids stored in the sucrose media, having localized coupling behavior, addition of $CaCl_2$ or $MgCl_2$ had no effect on the ATP formation onset lag, with or without pyridine. In the KCl-treated thylakoids, the PIP^+ ATP yields were also converted back (by 1 mM $CaCl_2$ or 30 mM $MgCl_2$) to being largely unaffected by pyridine addition (Table I). When 2 mM EGTA (a highly Ca^{2+} specific chelator) was included with the 100 mM KCl plus 30 mM $MgCl_2$ storage buffer (Figure 1D; Table I, line 5), or with the 100 mM KCl plus 1 mM $CaCl_2$ buffer (line 6), the ability of $MgCl_2$ or $CaCl_2$ to restore the thylakoid ATP onset lag to the pyridine-insensitive mode was blocked. Again, both the 12–15-flash extension of the ATP formation onset flash lag and the near doubling of the PIP^+ ATP yield by pyridine are indicative that the EGTA effect caused a delocalized coupling response. That Ca^{2+} may be the key ion for these effects was shown further by the fact that adding 4 mM $CaCl_2$ to the sample stored in 100 mM KCl, 30 mM $MgCl_2$, and 2 mM EGTA restored the localized proton gradient coupling pattern (Table I, line 7).

An indication that the Ca^{2+} effects are at a site within the membrane or at the lumenal surface rather than at the outside surface of the thylakoid was given by the finding that a lipid-soluble Ca^{2+} chelator, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), induced the pyridine effects when added to the pH 7.5 storage step even with the sucrose-stored thylakoids (which normally do not show the pyridine effects). TMB-8 at 25 μM caused a pyridine-dependent increase in the ATP formation onset of 11/10 flashes [30/38 flashes (−Pyr) and 33/43 flashes (+Pyr) for the controls; 34/44 flashes (−Pyr) and 45/54 flashes (+Pyr) for the TMB-8-treated sample]. Five millimolar $CaCl_2$ present with TMB-8 blocked the chelator effect mentioned above. The ATP yield per flash was not affected by the TMB-8 treatment, also showing that the lag extension was not due to inhibitory action.

A similar conclusion was reached when pH effects with the EGTA treatments were considered, in that 2 mM EGTA added to a low-salt storage medium induced, in the later phosphorylation assay, a significant pyridine effect indicative of delocalized coupling, but only when EGTA was given at acidic (pH 5.5) storage conditions. Typical data for 2 mM EGTA included in the usual low-salt storage buffer at pH 7.5 gave a lag extension due to 5 mM pyridine of 4/6 flashes

[35/46 (Pyr); 31/40 (−Pyr)] compared to 3/5 flashes (27/37 and 30/42) for the control (−EGTA), i.e., no effect of the EGTA. At a storage pH of 5.5 the low-salt-treated sample stored with 2 mM EGTA gave a pyridine-induced lag extension of 8/10 flashes (32/40 flashes for the −Pyr and 40/50 flashes for the 5 mM +Pyr sample); i.e., EGTA at low pH caused a significant pyridine sensitivity of the ATP formation onset lag. The ATP yield per flash was not affected by EGTA. Our present interpretation is that the acidic conditions (or the 30 mM $MgCl_2$ present in 100 mM KCl; Table I, line 5) provide sufficient screening of the EGTA $-COO^-$ charges to permit penetration of the chelator through the membrane, allowing it to mimic the effect of the membrane-permeable chelator TMB-8.

Discussion

Theoretical predictions, expectations based on past work (Vinkler et al., 1980; Ort et al., 1976; Nelson et al., 1971; Horner & Moudrianakis, 1983), and our extensive studies of pyridine effects on the ATP formation onset lag and postillumination ATP yield in thylakoids from either the low-salt or high-salt storage conditions (Beard & Dilley, 1986, 1987b; Beard et al., 1987) are all consistent with the conclusion that the energy-coupling proton gradient is localized in the low-salt-treated case and delocalized in the storage buffer containing 100 mM KCl. The delocalized case shows a pyridine-induced extension of the ATP formation onset lag parameters of about 12/20 flashes and about a 2-fold increase in the postillumination ATP yield (lines 2, 5, and 6, Table I). For all the results except treatment 4, which was low, the ATP yield per flash, a measure of the energy-coupling efficiency, was close to 0.40–0.45 (data not shown). Hence, the ATP formation onset lag length changes induced by the treatments were not due to significant alterations in energy-coupling efficiency.

The KCl effects have previously been interpreted as due to protons being shunted into the lumen during the buildup of the ΔpH needed for the energization threshold. The interpretation rests on a considerable body of data (Beard & Dilley, 1986, 1987a,b; Beard et al., 1987), including direct measurements of proton accumulation under phosphorylating conditions—and pyridine effects on them—with similar flash excitation regimes used to determine the ATP formation onset lags and postillumination phosphorylation [Table 3 of Beard et al. (1987)]. However, in the low-salt-treated case, the proton gradient can develop to the threshold and drive ATP formation without freely equilibrating with the lumen. Yet, with those low-salt-stored thylakoids, withholding ADP and P_i from the illumination stage (the traditional way to do the postillumination phosphorylation experiments) gives a greater proton accumulation and leads to equilibration of the proton gradient with the lumen, as shown by predictable pyridine effects on the traditional postillumination phosphorylation assay and on the proton accumulation in the flash train (Beard et al., 1987; Beard & Dilley, 1987b). Those effects and the fact that the 100 mM KCl (delocalizing) effect is reversed, either by a subsequent washing step in a buffer with 200 mM sucrose in place of 100 mM KCl (Beard & Dilley, 1987b) or by the $CaCl_2$ and $MgCl_2$ treatments reported here, suggest that some type of regulated, variable gating of the proton fluxes between a localized or a delocalized pathway can occur.

The key point of this paper is the finding that divalent cations, added to the 100 mM KCl containing storage buffer, caused the thylakoids to be kept in the state where pyridine did not extend the ATP formation onset lag; i.e., the localized proton gradient mode was retained as in the low-salt-stored case. Ca^{2+}, as $CaCl_2$, has its maximum effect at 1 mM, whereas 30 mM $MgCl_2$ was required to equal the 1 mM $CaCl_2$ effect. That difference, in addition to the observation that adding either 1 mM $CaCl_2$ or 30 mM $MgCl_2$ to the low-salt storage medium (it normally had 200 mM sucrose, 5 mM Hepes, and 3 mM $MgCl_2$) did not induce a pyridine effect on the ATP formation onset lag (Figure 2), suggests that the various ion effects are not due just to ionic strength (the ionic strength of 30 mM $MgCl_2$ is only 10% less than that of 100 mM KCl). Rather, the pattern suggests a specific ion effect.

That Ca^{2+} ions may play a specific role was indicated by the observation that when the Ca^{2+} chelator EGTA, at 2 mM, was present in the 100 mM KCl + 30 mM $MgCl_2$ media or in the 100 mM KCl + 1 mM $CaCl_2$ media, the pyridine effects (delocalized response) again were expressed (lines 5 and 6, Table I). Mg^{2+} ions cannot be the active agent in the former treatment because the 15-fold excess over EGTA provides adequate free Mg^{2+} beyond what may be chelated by 2 mM EGTA. However, if 30 mM $MgCl_2$ competitively displaced nonspecifically bound thylakoid Ca^{2+}—it is known that thylakoids contain high levels of Ca^{2+}, near 10–15 mM (Nobel, 1969; Nakatani et al., 1979)—such Ca^{2+} could then be available to interact with a putative high-affinity Ca^{2+} site associated with the proton flux gating. The 2 mM EGTA may be sufficient to chelate the Ca^{2+} pool strongly enough to block the Ca^{2+} from inducing the return to a localized gradient response to pyridine. The same argument could explain why 2 mM EGTA could block 1 mM $CaCl_2$ from reversing the delocalizing effect of the 100 mM KCl treatment. In keeping with the notion that the delocalization of the H^+ gradient, in these particular conditions, may be in response to the availability of Ca^{2+}, adding 4 mM $CaCl_2$ to the 100 mM KCl, 30 mM $MgCl_2$, and 2 mM EGTA storage medium switched the thylakoids back to the pyridine-insensitive, localized coupling mode (Table I, line 7). EGTA added to the pH 7.5 low-salt storage buffer did not affect the onset lags, with or without pyridine (see Results), so the effects are more subtle than a simple direct action of the chelator on an external membrane component such as CF_1. Adding 2 mM EGTA to the pH 5.5 low-salt storage buffer did result in converting the thylakoids to the pyridine-sensitive condition (cf. Results). We suggest that that reflects the ability of EGTA to penetrate the thylakoid more readily at pH 5.5 than at pH 7.5, thus chelating the Ca^{2+} suggested to be involved in an H^+ gating mechanism. Also, the membrane-permeable Ca^{2+} chelator TMB-8 (see Results) present in the pH 7.5 sucrose-based storage medium converted those thylakoids to the delocalized proton gradient behavior. Because EGTA, at acidic conditions, and the membrane permeable TMB-8 at pH 7.5 induced the delocalized coupling responses in the absence of 100 mM KCl, it is logical to suggest that Ca^{2+}-specific effects are involved in the H^+ gating rather than nonspecific ionic strength induced effects.

Hypothesis and Speculations. The following hypothesis is offered to explain the data and to provide a framework in which to design new experiments: Protons released in the PSI and PSII protolytic reactions are initially deposited in membrane-sequestered domains, through which they diffuse via a localized pathway to the CF_o–CF_1 complex. At some point, perhaps at the CF_o—but evidence is not available to specify this—a protein–protein interaction, cross-bridged between $-COO^-$ groups by Ca^{2+} ions, defines a "closed" gate when Ca^{2+} is in place. This would correspond to the H^+ gradient remaining localized, a characteristic of the high-sucrose, low-KCl-treated thylakoids. The 100 mM KCl incubation pro-

duces an "open" gate, either via a conformation change or by displacement of a critical Ca^{2+} ion, with the suggested $-COO^- K^+$ formation breaking the Ca^{2+} cross-bridges and consequently the protein–protein association. Protons from the localized domains would then equilibrate freely with the lumen. Figure 3 shows such a model as a working hypothesis. The assignment of one or more integral membrane proteins shown carrying the $-COO^-$ groups is speculative at this point. The possibility is obvious that one or more of the CF_o components are candidates for this proposed function. Nonetheless, protein carboxyl groups are logical candidates for Ca^{2+} binding sites, and the speculative model presented serves to focus attention on a type of gating function that, as a phenomenon, does not seem overly speculative, on the basis of the data presented here.

The Ca^{2+} gating hypothesis can also explain the observation that low-salt-stored thylakoids, which show localized proton gradient responses by the ATP formation onset lag and the PIP^+ ATP yield criteria, can also show the delocalized gradient response in the traditional postillumination phosphorylation (PIP^-) protocol (Beard et al., 1987; Beard & Dilley, 1987b). The latter can to be observed by using the same thylakoids and reaction media as for the former, except that ADP and P_i are added after the last flash of a flash train. The following points can be made:

(A) For the hypothesis to account for the data, the pH required to protonate the proposed $-COO^- {}^+Ca^+ {}^-OOC^-$ gate would have to be more acidic than the threshold pH needed to activate CF_1 and/or energize ATP formation. This could explain how a phosphorylating H^+ flux can occur in the localized pathway (Beard & Dilley, 1986, 1987b; Beard et al., 1987), but the gate does not open to the lumen, provided ongoing ATP formation can dissipate part of the protonmotive force, keeping the "proton pressure" below the putative threshold for gate opening. This also explains the lack of pyridine effect on the ATP formation onset lag and on PIP^+ ATP yield in the low-salt-treated thylakoids (Beard & Dilley, 1986, 1987b). Horner and Moudrianakis (1986) suggested a low p*K* group in the region of CF_o as a possible gate to the lumen, except they did not have information about divalent cation effects or reversibility in the gating response.

(B) Under basal electron flow, H^+ accumulation is greater than with coupling conditions because the H^+ efflux linked to ATP formation does not occur and the pH in the domains could become acidic enough to protonate the gate carboxyls, thereby inducing the displacement of the Ca^{2+} cross-bridge and allowing the proton gradient to equilibrate freely with the lumen. Hence, pyridine effects are predicted to be, and have been, observed on the "traditional" postillumination ATP yield (PIP^- in our terminology), even with the low-salt-stored thylakoids (Beard et al., 1987; Beard & Dilley, 1987b). This also accounts for the acidification of the lumen under basal electron flow conditions (regardless of the salts used for thylakoid storage), a clearly documented observation (Beard et al., 1987; Gaensellen & McCarty, 1971; Rottenberg et al., 1972), and rationalizes how, under coupling conditions, one can observe either a delocalized (Portis & McCarty, 1976) or a localized (see references cited above) proton gradient, depending on the salt concentration used and perhaps on other factors, freeze–thawing, osmotic stress, etc. De Kouchkovsky et al. (1986) have shown, with a different assay protocol than we used, that a variety of such treatments can shift the proton gradient coupling mode from a localized to a delocalized pattern. Hong and Junge (1983), using the neutral red pH indicator system, have reported that a freeze–thaw thylakoid treatment caused a shift from a localized to a delocalized proton response.

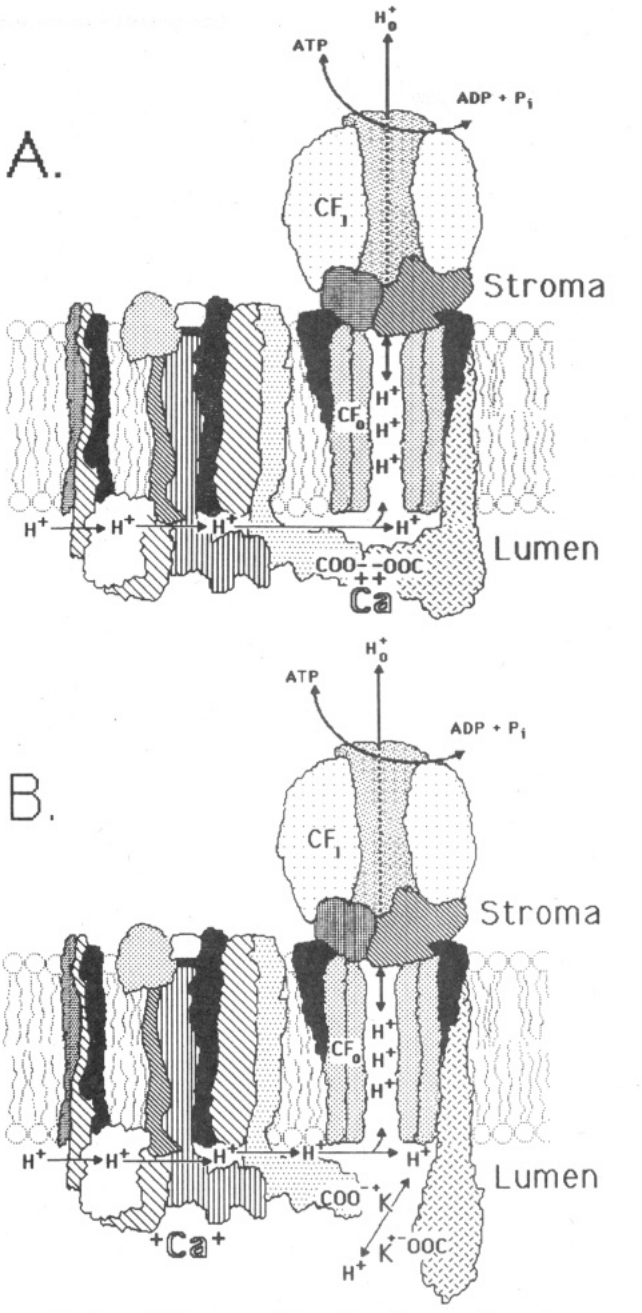

FIGURE 3: A model for a possible gating of proton fluxes between localized (A) or delocalized (B) energy-coupling gradients. (A) A portion of a thylakoid membrane is shown depicting, in a generalized and as yet speculative way, several intrinsic membrane proteins participating to form a localized proton diffusion domain from the proton-releasing reactions in H_2O oxidation and plastoquinol oxidation into the CF_o channel [cf. Dilley et al. (1987), Figure 4, and accompanying discussion therein for details]. Ca^{2+} ions are hypothesized to form a cross-bridge between adjacent protein $-COO^-$ groups to close a gated H^+ channel, although the cross-bridge could also form from tertiary structural parts of one polypeptide. (B) An open H^+ gate is shown owing to the putative Ca^{2+} ligand being displaced by K^+ ion, producing an H^+ equilibration pathway between the localized domains and the lumen. As mentioned in the text, a membrane-permeable Ca^{2+} chelator or EGTA, under acidic conditions, also results in the open gate mode, without the need for the 100 mM KCl treatment. We thank Dr. F. C. T. Allnutt for crafting the model with computer graphics.

(C) The proposed Ca^{2+}-regulated gating is consistent with our finding that when thylakoids were kept in a low-salt media and exhibit localized proton gradient coupling, the phenomenon was stable even under continuous illumination, steady-state ATP formation conditions [cf. Dilley et al. (1987) for a brief

description; S. M. Theg and R. A. Dilley, unpublished results]. However, a prediction of the hypothesis is that with sufficiently high rates of proton accumulation—beyond the capacity of the coupling reactions to utilize protons and after the buffering capacity of the localized domains is saturated—provided the domain pH drops acidic enough, the gate should open and proton equilibriation with the lumen should occur. It may also be that some proportion of the proposed gates may be either open or proton leaky, even under experimentally observed localized gradient coupling conditions, so that some acidification of the lumen could occur. Beard et al. (1987) have observed such subthreshold lumen acidification under localized coupling conditions at 5-Hz flash frequency but not at 1 Hz (cf. Table 3 of the above reference).

(D) The acid treatment during an acid–base ATP formation experiment (Uribe & Jagendorf, 1968) should, if acidic enough, protonate the gate carboxyls, opening the gate and permitting lumenally located protons (such as those associated with the succinic acid of the acid stage) to be utilized in driving ATP formation. A corollary of this, still to be tested, is that pretreatment such as high- or low-salt storage, may predispose the thylakoids to utilize lumenal protons more or less readily. The NaCl and low-tonicity treatments used by Uribe and Jagendorf (1968) may have had such effects, but this needs to be tested in view of the new developments and procedures now available.

While the hypothesis for Ca^{2+} regulation of a gating function for proton fluxes from localized to delocalized gradients certainly requires further testing, the concept of variable and regulatable gating of proton fluxes between proton gradients that are experimentally detected as being either membrane localized or delocalized seems to be on a firm foundation. One can inquire whether in intact chloroplasts, isolated or in situ, there is appreciable switching between the two energy-coupling modes. And, if so, what factors other than limitation of ADP and P_i to CF_1—or other ways of modulating the CF_1 activity—may be involved in regulating the proposed Ca^{2+} gating of H^+ fluxes. Perhaps varying ratios of K^+/Ca^{2+} or Mg^{2+}/Ca^{2+} in the lumen and/or the membrane phase could serve as regulatory signals. It should be noted that the high levels of Mg^{2+} and Ca^{2+} in intact chloroplasts [34 and 15 mM, respectively, with the K^+ level being near 51 mM; cf. Nakatani et al. (1979)] lead to the prediction that the Ca^{2+} gate proposed here normally should be in the closed configuration, promoting localized proton-gradient coupling. But, as mentioned above, certain conditions could cause a switching to the delocalized mode, making available additional carboxyl group buffer capacity in the lumen (Beard et al., 1987; Beard & Dilley, 1987b). As discussed in Dilley et al. (1987), renewed studies of thylakoid and intact chloroplast ion relations are clearly in order. The notion of gating the H^+ fluxes between localized and delocalized pathways was implicit in earlier results (Ort et al., 1976; Dilley et al., 1982; Dilley & Schreiber, 1984) and has recently been explicitly proposed by Horner and Moudrianakis (1986). The possible physiological significance of localized H^+ gradient coupling was discussed elsewhere (Dilley et al., 1987).

Registry No. ATP, 56-65-5; Ca, 7440-70-2; H^+, 12408-02-5; K, 7440-09-7; Mg, 7439-95-4.

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