Journal of Bioenergetics and Biomembranes, Vol. 26, No. 1, 1994

Evidence that the Intrinsic Membrance Protein LHCII in Thylakoids is Necessary for Maintaining Localized $\Delta\tilde{\mu}_{H^+}$ Energy Coupling

M. Renganathan[1] and Richard A. Dilley[1,2]

Received July 19, 1993; accepted September 13, 1993

This work tested the hypothesis that thylakoid localized proton-binding domains, suggested to be involved in localized $\Delta\tilde{\mu}_{H^+}$-driven ATP formation, are maintained with the involvement of several membrane proteins, including the LHCII (Laszlo, J. A., Baker, G. M., and Dilley, R. A. (1984) Biochim. Biophys. Acta 764, 160–169), which comprises about 50% of the total thylakoid protein. The concept we have in mind is that several membrane proteins cooperate to shield a localized proton diffusion pathway from direct contact with the lumen, thus providing a physical barrier to H^+ equilibration between the sequestered domains and the lumen. A barely mutant, *chlorina* f_2, that lacks Chl b and does not accumulate some of the LHCII proteins, was tested for its capacity to carry out localized-proton gradient-dependent ATP formation. Two previously developed assays permit clear discrimination between localized and delocalized $\Delta\tilde{\mu}_{H^+}$ gradient-driven ATP formation. Those assays include the effect of a permeable buffer, pyridine, on the number of single-turnover flashes needed to reach the energetic threshold for ATP formation and the more recently developed assay for lumen pH using 8-hydroxy-1,3,6-pyrene trisulfonic acid as a lumenally loaded pH-sensitive fluorescent probe. By those two criteria, the wild-type barley thylakoids revealed either a localized or a delocalized energy coupling mode under low- or high-salt storage conditions, respectively. Addition of Ca^{++} to the high-salt storage medium caused those thylakoids to maintain a localized energy-coupling response, as previously observed for pea thylakoids. In contrast, the *chlorina* f_2 mutant thylakoids had an active delocalized energy coupling activity but did not show localized $\Delta\tilde{\mu}_{H^+}$ energy coupling under any conditions, and added Ca^{++} to the thylakoid storage medium did not alter the delocalized energy coupling mode. One interpretation of the results is that the absence of the LHCII polypeptides produces a "leaky" pathway for protons which allows the $\Delta\tilde{\mu}_{H^+}$ gradient to equilibrate with the lumen under all conditions. Another interpretation is possible but seems less likely, that being that the absence of the LHCII polypeptides in some way causes the proposed Ca^{++} -gated H^+ flux site on the membrane sector (CF_0) of the energy coupling complex to lose its gating function.

KEY WORDS: Thylakoid proteins; proton gradients; localized energy coupling; ATP formation.

INTRODUCTION

In chloroplast thylakoid membranes energy coupling of the proton gradient to ATP formation has been suggested to occur either by a localized or a delocalized mechanism with the use of one or the other mode being dependent on ionic conditions (Beard and Dilley, 1988; Horner and Moudrianakis, 1986; Sigalat *et al.*, 1985). Although four groups have independently obtained evidence for ionic influences on the expression of localized or delocalized energy coupling (Beard and Dilley, 1988; Horner and

[1] Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1392.
[2] To whom correspondence should be addressed.

0145-479X/94/0200-0117$07.00/0

Moudrianakis, 1986; Sigalat *et al.*, 1985; Laasch, 1992), other groups have published evidence for the occurrence of both types of energy coupling (Graan *et al.*, 1981; Pick *et al.*, 1987; Laasch, 1992). The possibility that membranes can carry out localized $\Delta\tilde{\mu}_{H^+}$ energy coupling is at once an intriguing and a difficult idea to accept, for we are at a loss to explain just how a large $\Delta\tilde{\mu}_{H^+}$ can be constrained to a sequestered locus. The basic concept, though, is so important to biology that the quest must go on to elucidate the mechanism(s) involved in localized energy coupling or to explain how the manifold and diverse observations which have suggested localized energy coupling can be better understood by other possible explanations.

Experiments with monovalent and divalent cation levels and Ca^{++} chelators support the notion that thylakoids can be reversibly switched between localized and delocalized energy coupling modes by factors which perturb Ca^{++} binding to the CF_0H^+ channel (Chiang and Dilley, 1987; Chiang *et al.*, 1992; Dilley 1991). Recent results in measuring lumen pH with the fluorescent dye pyranine (Renganathan *et al.*, 1993), showed that under localized coupling conditions the lumen pH does not drop below pH 7, whereas the sequestered domains must have been at least as acidic as pH 5.8 to accommodate the thermodynamic requirements for energizing ATP formation. What is the structural basis inherent in the thylakoid membrane that can maintain a pool of localized H^+ ions at such an acidic level compared to the lumen? This question does not yet have a clear answer, but one interesting possibility is in the already-documented phenomenon of sequestered, metastable ionizable groups associated with several thylakoid proteins [Prochaska and Dilley, 1978; Laszlo *et al.*, 1984; Allnutt *et al.*, 1989; Tandy *et al.*, 1982; see Dilley *et al.* (1987) for a review].

Laszlo *et al.* (1984) identified up to eight thylakoid proteins as having low pK_a lysine groups in the sequestered domains, including the LHCII[3] polypeptides, the three extrinsic PSII proteins (Allnutt *et al.*, 1989), and the 8-kDa CF_0 subunit (Tandy *et al.*, 1982). Theg *et al.* (1988) showed that the metastable domain protons are "... the first in line in the flow of H^+ ions involved in the energetic H^+ gradient driving ATP formation."

A model coming out of those studies proposed that the putative localized protons are sequestered in spaces between the lipid bilayer and the overlaying lumen-exposed parts of those several thylakoid proteins (Laszlo *et al.*, 1984; Allnutt *et al.*, 1989; Dilley *et al.*, 1987). This model has its difficulties. For instance, it is a problem to visualize how the protons, which may have to diffuse over some distance, could be so thoroughly occluded by the proteins. It should be emphasized that the model we envision for the sequestered domain posits not a space at the interface between the membrane and the lumen, but a space behind a barrier (such as the overlying protein mass on the lumen side). Protons released at an interface with the lumen—and at a high electrochemical potential—are not expected to have the long residence times observed in our past work for the "domain protons" (Beard and Dilley, 1988; Laszlo *et al.*, 1984). Theoretical considerations predict this (Nagle and Dilley, 1986), and time-resolved H^+ diffusion data show that either proteinaceous or phospholipid interfaces do not constrain protons from equilibration with the bulk phase in submillisecond times [cf. Gutman and Nachliel (1990) for a review]. Hence, to account for observations with thylakoids which show metastable, sequestered protons having long residence times [tens of minutes (Laszlo *et al.*, 1984; Baker *et al.*, 1981; Theg *et al.*, 1982; Johnson *et al.*, 1983; Pfister and Homann, 1986)], there must be a barrier or a shield which occludes the metastable protons from equilibration with the bulk phase. The lumen-exposed parts of membrane-associated extrinsic (e.g., the PSII-associated polypeptides with M_r of 18, 22 and 33 kDa) or intrinsic (e.g., the LHCII and the 8-kDa CF_0 polypeptides) proteins could play that role and provide a H^+ diffusion pathway into the CF_0H^+ channel, although structural details for this remain to be elucidated.

Others who consider localized coupling have suggested that the localized H^+s may be tightly bound in the (mobile) redox carriers and delivered to the CF_0–CF_1 complex for collisional interaction exchange which would not require a long-distance occluded diffusion pathway (Pick *et al.*, 1987) or that nonbilayer lipid structures may provide the sequestering structure (V. Skulachev, personal communication). The question remains open.

[3] Abbreviations used: DCCD, dicyclohexylcarbodiimide; DCMU, *N'*-(3,4-dichlorophenyl)-*N,N*-dimethylurea; DQH_2, duraquinol; DTT, dithiothreitol; HPTS, 8-hydroxy-1,3,6-pyrene trisulfonic acid; LHCII, light-harvesting complex; MV, methylviologen; PSI, PSII, photosystem I or II, respectively; PYR, pyridine.

A possible approach to studying the question seems available in the use of mutants lacking, for instance, some of the LHCII polypeptides. Our reasoning was that, if the LHCII—being normally near 50% of the total thylakoid protein—provided important structural motifs to help sequester the domain protons (and perhaps provide H bonding or H^+ charge relay sites in the H^+ diffusion pathway), then the deletion of such a protein class could result in a "leaky" H^+ pathway, causing obligatory delocalization of the H^+ gradient.

We chose for this work the barley *chlorina* f_2 2800 mutant (kindly supplied by Dr. D. J. Simpson, Carlsberg Laboratories) which lacks Chl b and is deficient in the LHCII polypeptides (Machold *et al.*, 1979; Ryrie, 1983; White and Green, 1988) particularly LHCII b and d (Peter and Thornber, 1991). The absence of the LHCII from the thylakoid membranes does not affect the normal functioning of the two photosystems (Boardman and Highkin, 1966), and the *chlorina* f_2 2800 has an added point that even though it is deficient in the LHCII polypeptides, it forms rather normal grana (Goodchild *et al.*, 1966; Simpson, 1979; Burke *et al.*, 1979; Ouijja *et al.*, 1988), having similar gross structural aspects compared to normal thylakoids.

Thylakoids isolated from wild type (*donaria*) and *chlorina* f_2 barley were assayed for their capacities to carry out localized and delocalized $\Delta\tilde{\mu}_{H^+}$ energy coupling, using the previously developed methods to shift the coupling response between the two modes (Beard and Dilley, 1988; Chiang and Dilley, 1987). We also determined the magnitude of the ΔpH gradient between the lumen and the external phase under coupled and basal electron transport conditions using a new fluorescent dye method (Renganathan *et al.*, 1993), as an additional parameter to verify the occurrence of localized or delocalized energy coupling.

MATERIALS AND METHODS

Plant Growth and Thylakoid Isolation

Barley wild type (*donaria*) and barley mutant plants (*chlorina* f_2 2800), kindly supplied by Dr. D. J. Simpson of the Carlsberg Laboratories, were grown in a greenhouse with supplemental light (400 W high-pressure sodium lamp) in 12 h light/dark cycles and harvested 3–4 weeks after sowing. Broken chloroplasts were prepared from these plants as described in Beard and Dilley (1988) and suspended either in 200 mM sucrose, 5 mM Hepes-KOH (pH 7.5), 3 mM $MgCl_2$ (or 10 and 25 mM $MgCl_2$ as indicated in the legend of Table I), and 0.1% defatted bovine serum albumin (low-salt storage), or in 130 mM sucrose, 50 mM KCl, 5 mM Hepes-KOH (pH 7.5), and 3 mM $MgCl_2$, 0.1% defatted bovine serum albumin (high-salt storage).

Flash-Induced ATP Formation

Single-turnover flash-induced ATP formation was measured using the luciferin-luciferase enzyme complex as described by Beard and Dilley (1988). At time zero, 15 μM chl thylakoids were added to the reaction medium consisting of 50 mM Tricine-KOH (pH 8.0), 10 mM sorbitol, 3 mM $MgCl_2$ (or 10 mM $MgCl_2$ as indicated in Table I legend), 5 mM KH_2PO_4, 0.2 mM ADP, 0.2 mM MV, 5 mM DTT, 400 nm nonactin and 5 μM diadenosine pentaphosphate (to inhibit adenylate kinase) in the presence or absence of 5 mM pyridine. For DQH_2 to MV supported ATP formation, 0.5 mM reduced duroquinol and 5 μM DCMU were added in addition to the above-mentioned reagents. Duroquinone was reduced in ETOH (stock concentration 50 mM) by adding a few milligram of borohydride, and the excess borohydride was removed by adding 5 μl per ml of concentrated HCl. At 2 min, 10 μl of luciferin-luciferase enzyme from a stock of 1.5 ml/vial of the Pharmacia ATP kit [cf. Beard and Dilley (1988)] was added. At 3 min, a train of 150 single-turnover flashes at 5 Hz frequency was fired to energize ATP formation. At the end of each assay, the signal was calibrated by addition of standard ATP. The end of the ATP formation onset lag, indicating the reaching of the energetic threshold, was determined as in Beard and Dilley (1988) by counting the number of flashes required to obtain the first detectable rise in the ATP signal and the flashes corresponding to the intersection of the extrapolated line of the rising ATP signal with the horizontal.

Lumen pH Determination

The lumen pH was estimated using the fluorescent probe HPTS (8-hydroxy-1,3,6-pyrenetrisulfonic acid, common name, pyranine) as in Renganathan *et al.* (1993) except that in some experiments sucrose replaced sorbitol in the buffers. The dye was loaded into the lumen at 0.5 mM HPTS and with chloroplast

Table I. Effect of pyridine on ATP Formation Onset Lags in Barley Wild Type and the *chlorina* f_2 Mutant Thylakoids[a]

Thylakoid type	Storage condition	Pyridine	Flash lag	Flash yield, nmol ATP (μmol chl · flash)$^{-1}$	Flash lag (+)–(−) pyridine	Deduced energy coupling mode
			A. Normal storage media			
Wild type	Low salt	−	47/56 ± 2/5	0.45 ± 0.07		
Wild type	Low salt	+	48/61 ± 1/3	0.49 ± 0.04	1/5	Loc.
Wild type	High salt	−	53/67 ± 1/1	0.51 ± 0.02		
Wild type	High salt	+	62/78 ± 0/3	0.43 ± 0.05	9/11	Deloc.
Mutant	Low salt	−	56/71 ± 3/2	0.52 ± 0.10		
Mutant	Low salt	+	71/81 ± 3/1	0.44 ± 0.15	15/10	Deloc.
Mutant	High salt	−	74/93 ± 4/1	0.26 ± 0.00		
Mutant	High salt	+	95/106 ± 1/3	0.27 ± 0.10	11/13	Deloc.
			B. Normal storage medium +10 mM $MgCl_2$ or 25 mM $MgCl_2$			
Wild type	Low salt +10 mM $MgCl_2$	−	31/41 ± 1/1	0.77 ± 0.1		
Wild type	Low salt +10 mM $MgCl_2$	+	31/42 ± 0/2	0.80 ± 0.04	0/1	Loc.
Mutant	Low salt +10 mM $MgCl_2$	−	23/32 ± 2/3	0.85 ± 0.1		
Mutant	Low salt +10 mM $MgCl_2$	+	33/43 ± 2/3	0.91 ± 0.2	10/11	Deloc.
Mutant	Low salt +25 mM $MgCl_2$	−	28/37 ± 1/1	1.18 ± 0.1		
Mutant	Low salt +25 mM $MgCl_2$	+	38/47 ± 2/2	1.28 ± 0.1	10/10	Deloc.

[a] Broken chloroplasts (thylakoids) from barley wild type (*donaria*) and barley LHCII-less mutant (*chlorina* f_2) were prepared and stored as in Materials and Methods. Single-turnover flash-induced ATP formation was measured by monitoring luciferin-luciferase fluorescence emission as described in Materials and Methods. The criteria for measuring the flash lag are explained in Methods. The column on the right indicates the type of energy coupling response, either localized or delocalized as judged by the response to the presence of the pyridine as described in Beard and Dilley (1988). In Experiment A the low-salt storage medium contained 3 mM $MgCl_2$ as in previous work (Beard and Dilley, 1988). The values listed are averages of generally four repeats. In Experiment B, the 10 mM or 25 mM $MgCl_2$ was used in the low-salt storage medium and in the phosphorylation assay medium. Four, or sometimes three, repeats of each measurement were averaged. The higher ATP yield per flash for Part B was the result of a more intense flash excitation than used in Experiment A.

membranes at 1 mg chl ml^{-1} at pH 6.0 for 30 min at 15°C. The dye loading medium was 200 mM sorbitol (or sucrose, as indicated), 20 mM Mes-KOH (pH 6.0), 3 mM $MgCl_2$, and 20 mM KCl. After the 30 min loading, the thylakoids were kept at 4°C in the same medium. Just prior to the pH assay aliquots were centrifuged, suspended in pH 8.5 medium of 200 mM sorbitol (or sucrose, as indicated), 20 mM Tricine-KOH (pH 8.5), 3 mM $MgCl_2$, and 20 mM KCl, centrifuged again, and finally suspended in a similar assay medium but with 0.1 mM methyl-viologen, 0.2 mM ADP, 5 mM K_2HPO_4, 100 nM nonactin, and with 10 μM chl. The assay was carried out in an SLM DMX-1000 spectrofluorimeter at 10°C and the dye fluorescence emission was monitored at 511 nm using 450 and 405 nm excitation as in Renganathan *et al.* (1993). Calibration of the lumen pH dependence of the fluorescence was done as in Renganathan *et al.* (1993). Heat-filtered red actinic light (continuous illumination for 20 s to 1 min) was directed onto the cuvette to drive electron/proton transfer.

RESULTS

The *chlorina* f_2 plants showed the expected phenotype (pale green leaves) and the isolated thylakoids had no Chl b, determined by an HPLC assay as in Pfündel and Dilley (1993) (data not shown). The experiments used to test for localized or delocalized energy coupling in the mutant and wild-type barley thylakoids began with measurements of permeable buffer (pyridine) effects on the ATP formation onset lag in single-turnover flash trains as in earlier work (Beard and Dilley, 1988;

Chiang and Dilley, 1987). In the earlier work with pea or spinach thylakoids, low-salt stored thylakoids showed no significant effect of 5 mM pyridine on the number of flashes required to reach the energization threshold (valinomycin or nonactin plus K^+ present to suppress the electric field contribution to the protonmotive force). Wild-type barley thylakoids stored in a low-salt medium gave a similar lack of response to added pyridine as seen in Table I. In wild-type barley thylakoids, suspended in a high-salt storage medium [storage only, the ATP formation assays were always done in an identical medium (cf. legend of Table I)], the presence of pyridine gave about a 10-flash extension of the ATP formation onset lag (Table I), an effect similar to what is observed in pea thylakoids assayed under those conditions. That response to pyridine is taken as evidence for a delocalized energy coupling $\Delta\tilde{\mu}_{H^+}$ gradient.

The *chlorina* f_2 thylakoids showed only a delocalized energy coupling response in the flash-driven ATP formation assay whether stored in low- or high-salt medium (Table I). In the mutant thylakoids, the ATP onset lag was extended by about 10 to 15 flashes by 5 mM pyridine in either the low- or high-salt stored condition. That is close to the same pyridine effect observed in the wild-type high-salt storage case (Table I). This pattern implies that the *chlorina* f_2 thylakoid membranes do not sequester protons in the putative localized domains suggested to be present in normal, low-salt stored thylakoids.

Table IA shows that the mutant and wild-type thylakoids have similar efficiencies of ATP formation with values near 0.4–0.5 nmol ATP (μmol chl · flash)$^{-1}$ for low-salt storage, typical of results reported earlier with pea thylakoids (Beard and Dilley, 1988; Chiang and Dilley, 1987). The mutant plant thylakoids stored in high-salt medium had a lower but still respectable yield of near 0.3 nmol (μmol chl · flash)$^{-1}$. Combined with the observed longer ATP formation onset lag in the mutant, high-salt storage case, it suggests that those membranes may have been slightly more leaky to H^+ ions than the wild type or the mutant thylakoids stored in low salt.

This brings up the question of whether the mutant plant thylakoids have any gross morphological features different from the wild type which could influence ion fluxes or energy coupling. It is known, for instance, that grana stacking is influenced by surface charges, some of which can be contributed by some of the LHC subunits (Burke *et al.*, 1979). That issue prompted several studies of thylakoid structure in mutants lacking some of the LHCII subunits, and the ultrastructure data clearly show that mutants such as the *chlorina* f_2 have normal grana stacking (Goodchild *et al.*, 1966; Simpson, 1979; Burke *et al.*, 1979; Ouijja *et al.*, 1988). While it is not evident that unstacked grana would respond any differently in ATP formation assays, we were concerned enough to do controls at sufficiently high Mg^{++} concentrations to ensure that the grana would remain stacked throughout the isolation and assay procedures.

One report indicated that the mutant showed normal grana stacking only when 10 mM $MgCl_2$ was used in the isolation and assay media (Burke *et al.*, 1979) rather than the usual 3 mM $MgCl_2$. In the latter case, Burke *et al.* (1979) reported that significant unstacking occurred in isolated thylakoids when 3 mM $MgCl_2$ was used for the experimental assays. However, it should be noted that those authors used a thylakoid isolation medium with no added Mg^{++} (0.4 M sorbitol, 0.1 M Na-Tricine, 0.05 M Na ascorbate, and 0.25% BSA). Tricine has significant Mg^{++}-chelating capacity and at 0.1 M it might be expected to compete for thylakoid-bound Mg^{++}. Such an effect could complicate the ultrastructural analysis. Nonetheless, we compared our normal 3 mM $MgCl_2$ conditions with 10 mM and 25 mM $MgCl_2$ added both to thylakoid storage and to the ATP formation assays. Table IB shows that the mutant thylakoids still expressed a delocalized response with 10 mM or 25 mM $MgCl_2$ in place of 3 mM $MgCl_2$. Either there is little tendency for the thylakoids to unstack under our conditions or the degree of stacking is not a critical factor in determining localized or delocalized energy coupling.

Another, more direct assay for delocalized or localized energy coupling is the measurement of the lumen pH under the various conditions. We used the HPTS fluorescence method recently worked out for thylakoids (Renganathan *et al.*, 1993) and measured the fluorescence signals in the mutant and wild type barley thylakoids under coupled and basal conditions. The results, shown in Table II, indicate that the *chlorina* f_2 mutant thylakoids stored in low-salt medium and assayed under coupling conditions had a Δ pH ≥ 2.5, indicative of delocalized $\Delta\tilde{\mu}_{H^+}$ energy coupling, whereas the wild type thylakoids had a ΔpH of about 1 unit. A ΔpH of 1 unit is too small to account for the energy requirements of delocalized

Table II. Lumen-to-Outside ΔpH Values iin Barley Wild Type and *chlorina* f_2 Thylakoids under Coupled and Basal Conditions[a]

	ΔpH	
	Coupled	Basal
Wild type	0.97 ± 0.03, $n = 3$	≥ 2.5,[b] $n = 3$
Chlorina f_2 mutant	≥ 2.5,[b] $n = 4$	≥ 2.5, $n = 3$

[a] The ΔpH values were determined using the HPTS fluorescence technique as described in Materials and Methods. The dye was loaded at pH 6.0 and the fluorescence assays were done at pH 8.5. In the experiment shown, sucrose was the osmotic agent used both in the storage and assay media, but in other experiments sorbital was used as in Renganathan *et al.* (1993), and gave similar results.

[b] The lumen pH cannot be precisely determined when the pH approaches 6 owing to the loss of pH-dependent fluorescence changes in the pH 6 region (Renganathan *et al.*, 1993).

energy coupling, as discussed in Renganathan *et al.* (1993); thus, it is evident that those membranes were carrying out the localized $\Delta\tilde{\mu}_{H^+}$ energy coupling. Under basal conditions (−ADP) both mutant and wild type thylakoid samples showed ΔpH values ≥ 2.5. The ΔpH pattern observed in the wild type barley is similar to that observed in pea thylakoids as reported in Renganathan *et al.* (1993), but the mutant gave only the large ΔpH. The actual value of the lumen pH, when the ΔpH was indicated as ≥ 2.5, cannot be accurately determined when the external pH is 8.5 because the dye changes become quite insensitive to pH at pH values near or below 6 [cf. Renganathan *et al.* (1993)].

The question arises as to whether the absence of LHCII in the *chlorina* f_2 mutant affects the energy coupling patterns of protons derived from PSII water oxidation differently than H^+ release from PQH_2 oxidation by the cyt b_6f complex. The reasoning behind this question has to do with the notion that in dark-adapted chloroplasts with the usual low level of LHC phosphorylation, the mobile LHCII is believed to associate closer to PSII units than PSI units (Staehelin and Arntzen, 1983). Thus, it could be imagined that the absence of the LHCII proteins might have a greater effect on PSII- than PSI-derived protons. We tested this by measuring the pyridine effect on the ATP formation onset lag using duroquinol (+DMCU) as a PSI electron donor to the b_6f complex (Izawa and Pan, 1978). Table III shows that the pattern of onset lags was essentially the same for the $DQH_2 \rightarrow$ methylviologen system as for the $H_2O \rightarrow$ methylviologen system shown in Table I. The ATP yields per flash were, predictably, somewhat lower than those for the PSII + PSI system, owing to the fact that the PSI system has a lower H^+/e^- (or H^+/single-turnover flash) ratio; i.e., Hangarter *et al.* (1987) found an $H^+/e^- = 2.7 \pm 0.3$ for $H_2O \rightarrow$ methylviologen and 1.7 ± 0.2 for the photosystem I-dependent DQH_2 donor system.

In earlier work we showed that adding 0.5 to 1.0 mM $CaCl_2$ to the high-salt storage medium of pea thylakoids blocked the tendency of the 100 mM KCl in the storage medium to cause the delocalized energy coupling pattern (Chiang and Dilley, 1987). Those data and other results (Chiang *et al.*, 1992) were interpreted as owing to Ca^{++} ions normally present in thylakoids and retained in the low-salt medium or the 0.5–1.0 mM $CaCl_2$ added to the high-salt storage medium, interacting with the

Table III. ATP Formation Onset Flash Lag in the PSI Electron Transport System (Duraquinol → Methyl Viologen) for Wild Type and Mutant Barley Thylakoids[a]

Thylakoid type	Storage condition	Pyridine	Flash lag	Flash yield, nmol ATP (μmol chl · flash)$^{-1}$	Flash lag (+)–(−) pyridine	Deduced energy coupling mode
Wild type	Low salt	−	$50/64 \pm 0/0$	0.37 ± 0.00		
Wild type	Low salt	+	$52/66 \pm 1/2$	0.31 ± 0.04	2/2	Loc.
Mutant	Low salt	−	$58/71 \pm 0/0$	0.42 ± 0.03		
Mutant	Low salt	+	$69/82 \pm 2/1$	0.48 ± 0.03	11/11	Deloc.
Wild type	High salt	−	47/63	0.31		
Wild type	High salt	+	63/79	0.24	15/16	Deloc.
Mutant	High salt	−	75/90	0.21		
Mutant	High salt	+	94/118	0.21	18/18	Deloc.

[a] The conditions were similar to those given in Table I except that electron/proton transport was driven by the PSI-specific $DQH_2 \rightarrow$ MV system with DCMU added to block PSII function as described in Materials and Methods. Each assay was done a minimum of three times for the low-salt conditions and twice for the high-salt conditions. The flash lag criteria are as explained in Methods.

CF_0H^+ channel and functioning as a regulatory factor which controls H^+ ion spillover from the sequestered domains into the lumen. However, the present results raise an alternative interpretation; namely, that the high-salt storage conditions reported earlier (Beard and Dilley, 1987) or the effects of Ca^{++} chelator addition to a low-salt storage medium (Chiang and Dilley, 1988)—both leading to the delocalized $\Delta\tilde{\mu}_{H^+}$ coupling mode—could have been mediated by perturbations of the LHCII proteins. Prochaska and Gross (1977) and Davis and Gross (1975) showed that the LHCII proteins have high-affinity Ca^{++}-binding sites; thus, it is possible that these proteins, being about 50% of the total thylakoid protein, could have an influence on Ca^{++} levels in the membrane. For example, is it possible that the absence of the LHCII could lead to a generally lower amount of Ca^{++} retained in the isolated thylakoids and that could result in the inducing of delocalized coupling, in analogy to treatment of thylakoids with Ca^{++} chelators (Chiang and Dilley, 1987)? To test this, we added 5 mM $CaCl_2$ to both the wild type and the mutant thylakoid storage medium and measured the pyridine effect on the ATP formation onset lag. Table IV shows that Ca^{++} added to the wild type high-salt storage medium caused those thylakoids to revert to a localized coupling pattern (i.e., only a slight pyridine effect on the flash number required to reach the energization threshold). However, the mutant plant thylakoids in the low-salt storage medium without and with 5 mM $CaCl_2$ added gave the delocalized coupling response in both cases. Hence, the delocalized coupling response consistently seen in thylakoids from the *chlorina* f_2 mutant was not caused simply by insufficient Ca^{++}.

DISCUSSION

The absence of two of the LHCII proteins [subunit b and d are absent (Peter and Thornber, 1991)] in the *chlorina* f_2 barley mutant is shown in this work as being clearly correlated with the inability of those thylakoid membranes to carry out localized $\Delta\tilde{\mu}_{H^+}$ energy coupling. Why should a light-harvesting complex protein, having no direct association with the H^+ releasing redox reactions of PSII [certainly not a functional role, witness the *chlorina* f_2 mutant having active PSII function (Boardman and Highkin, 1966; Ouijja *et al.*, 1988)], have such a pronounced effect on H^+ diffusion pathways in the energy coupling mechanism? We can speculate along three lines. (A) The LHCII proteins may directly provide an essential part of the putative localized H^+ diffusion pathway between the redox reactions which release H^+ ions and the CF_0–CF_1 complex. Jahns *et al.* (1988) suggested something along this line, but they do not comment on the question of localized H^+ conduction pathways. (B) The absence of the LHCII causes a general perturbation in the thylakoid, which, for unspecified reasons, results in observing only delocalized $\Delta\tilde{\mu}_{H^+}$ coupling, or (C) the LHCII, being somewhat mobile, participates in carrying H^+ ions, sequestered in its structure, from points of release by the redox reactions to or near the CF_0–CF_1 (Pick *et al.*, 1987).

It is important to note that the *chlorina* f_2 mutant, while lacking LHCII b and d subunits (Peter and Thornber, 1991), retains normal grana stacking (Goodchild *et al.*, 1966; Simpson, 1979; Burke *et al.*, 1979; Ouijja *et al.*, 1988). One report (Burke *et al.*, 1979) suggested that the isolated mutant thylakoids

Table IV. Lack of Effect of 5 mM Ca^{2+} Addition to Low-Salt Stored Mutant Thylakoids in Converting Delocalized Coupling to Localized Coupling.

Thylakoid type	Storage condition	Pyridine	Ca^{2+}	Flash lag	Flash yield, nmol ATP (mg chl · flash)$^{-1}$	Flash lag (+)–(−) pyridine	Deduced energy coupling mode
Mutant	Low salt	−	−	50/69 ± 4/4	0.39 ± 0.04		
Mutant	Low salt	+	−	62/79 ± 5/4	0.36 ± 0.01	12/10	Deloc.
Mutant	Low salt	−	+	62/77 ± 3/2	0.24 ± 0.05		
Mutant	Low salt	+	+	74/89 ± 2/2	0.22 ± 0.02	12/12	Deloc.
Wild type	High salt	−	−	36/52 ± 0/2	0.29 ± 0.03		
Wild type	High salt	+	−	49/63 ± 2/1	0.30 ± 0.03	13/11	Deloc.
Wild type	High salt	−	+	41/55 ± 2/2	0.31 ± 0.01		
Wild type	High salt	+	+	43/59 ± 3/1	0.35 ± 0.02	2/4	Loc.

[a] The ATP formation onset lags were measured as in Table I and explained in Methods. Calcium chloride at 5 mM was added to the storage buffer of the indicated treatments but not to the assay medium. Each assay condition was repeated a minimum of three times.

did not have well-developed grana stacks in 3 mM $MgCl_2$ but they did in 10 mM $MgCl_2$. However, we found no difference in the mutant energy coupling response in 3 mM versus 10 mM $MgCl_2$ (Table IB). It should be noted than we used 3 mM $MgCl_2$ in the isolation and the storage medium, whereas in (Burke *et al.*, 1979) the authors isolated membranes in medium with no $MgCl_2$ added and with 0.1 M Tricine-NaOH, a strong Mg^{++} chelator.

In considering point (A), the LHCII proteins and several others have been shown to contribute significantly to the sequestered domain–metastable amine–buffering array (Laszlo *et al.*, 1984; Allnutt *et al.*, 1989). The role of the proteins carrying such groups has been hypothesized to provide the necessary physical barrier to maintain the sequestered H^+ diffusion pathway (Dilley *et al.*, 1987), thus occluding the domain protons from ready access to the lumen. Perhaps the absence of the LHCII protein produces a leak in the H^+ diffusion pathway. In such a model, the LHCII must have lumenally protruding parts of the protein which interact closely enough with both a H^+ donor and an acceptor neighbor to maintain the occluded H^+ diffusion pathway. Structural studies indicate that the LHCII polypeptides have a considerable mass of lumenally exposed protein (Peter and Thornber, 1991; Karlin-Neumann *et al.*, 1985; Kühlbrandt and Wang, 1991) and the amino acid sequences of those regions suggest there are numerous buffering groups (five carboxylic and three lysine per LHCII monomer) and other H-bonding groups among the 58 amino acids predicted to be positioned on the lumenal side (Karlin-Neumann *et al.*, 1985).

The LHCII proteins recently have been implicated with H^+ diffusion events associated with protons released in PSII water oxidation (Jahns *et al.*, 1988; Jahns and Junge, 1990) using DCCD (dicyclohexylcarbodiimide) as a reagent to influence proton transport as assayed by neutral red changes. However, as shown here, the absence of the LHCII b and d subunits in the *chlorina* f_2 mutant also creates a delocalized-only energy coupling response for H^+ gradients derived from the $DQH_2 \rightarrow$ methylviologen PSI-specific electron/ proton transport system (Table III). Therefore, it seems that the effects reported by Junge's group cannot be attributed to aberrations in H^+ transport solely in the region of PSII. In fact, the data of Jahns *et al.* (1988) are equally consistent with the DCCD effect influencing proton movement in sequestered domains rather than specifically causing a H^+ ion "short-circuit" to the outside of the membrane only for PSII-derived protons (as proposed by those authors).

Some of the carboxylic groups of LHCII were shown by Jahns and Junge (1990) to be labeled by [^{14}C]DCCD, implicating those groups as being in a hydrophobic environment. This is reminiscent of well-known dual aspects of the 8-kDa CF_0 subunit, being involved in H^+ conduction in the hydrophobic region and having a DCCD-reactive carboxyl group. Hence, it heightens the interest in, and possibilities for, LHCII functioning as part of a proton conduction pathway which is occluded from the lumen bulk phase. However, this is still a speculative issue, as there is so little understanding of how protons move through the membrane. Even so, it is of particular relevance that DCCD treatment of thylakoids greatly perturbs H^+ flow, blocking lumenal H^+ deposition, as monitored by the neutral red probe (Jahns and Junge, 1990), without significantly affecting electron transport. Horton's group also reported that DCCD blocked lumenal H^+ accumulation, assayed by the 9-amino acridine fluorescence change (Ruban *et al.*, 1992).

The above data, while indicating the involvement of LHCII in H^+ interactions in bioenergetics, does not yet permit a clear selection of alternatives (A) to (C) listed above. Further work is needed to elucidate the intriguing role which LHCII plays in thylakoid–proton interactions, and indeed, to answer the deeper question of how thylakoid $\Delta\tilde{\mu}_{H^+}$ gradients are kept localized under some conditions and reversibly switched to delocalized gradients under other conditions.

ACKNOWLEDGMENTS

The authors thank Janet Hollister for excellent assistance in preparation of the manuscript. This work was supported in part from grants from the U.S. Department of Energy and the U.S.D.A.

REFERENCES

Allnutt, F. C. T., Atta-Asafo-Adjei, E., and Dilley, R. A. (1989). *J. Bioenerg. Biomembr.* **21**, 535–551.

Baker, G., Bhatnagar, D., and Dilley, R. A. (1981). *Biochemistry* **20**, 2307–2315.

Beard, W. A., and Dilley, R. A. (1988). *J. Bioenerg. Biomembr.* **20**, 129–154.

Boardman, N. K., and Highkin, H. R. (1966). *Biochim. Biophys. Acta* **126**, 189–199.
Burke, J. J., Steinback, K. E., and Arntzen, C. J. (1979). *Plant Physiol.* **63,** 237–243.
Chiang, G., and Dilley, R. A. (1987). *Biochemistry*, **26,** 4911–4916.
Chiang, G., Wooten, D., and Dilley, R. A. (1992). *Biochemistry* **31**, 5808–5819.
Davis, D. J., and Gross, E. L. (1975). *Biochim. Biophys. Acta* **387**, 557–567.
Dilley, R. A. (1991). *Curr. Top. Bioenerg.* **16**, 265–318.
Dilley, R. A., Theg, S. M., and Beard, W. A. (1987). *Annu. Rev. Plant Physiol.* **38**, 347–389.
Goodchild, D. J., Highkin, H. R., and Boardman, N. K. (1966). *Exp. Cell Res.* **43**, 684–688.
Graan, T., Flores, S., and Ort, D. R. (1981). In *Energy Coupling in Photosynthesis* (Selman, B. R., and Selman-Reimer, S., eds.), *Developments in Biochemistry*, Vol. 20, Elsevier North-Holland, New York, pp. 25–34.
Gutman, M., and Nachliel, E. (1990). *Biochim. Biophys. Acta* **1015**, 391–414.
Hangarter, R. P., Jones, R. W., Ort, D. R., and Whitmarsh, J. (1987). *Biochim. Biophys. Acta* **890**, 106–115.
Horner, R. D., and Moudrianakis, E. N. (1986). *J. Biol. Chem.* **261**, 13408–13414.
Izawa, S., and Pan R. L. (1978). *Biochem. Biophys. Res. Commun.* **83**, 1171–1177.
Jahns, P., and Junge, W. (1990). *Eur. J. Biochem.* **193**, 731–736.
Jahns, P., Polle, A., and Junge, W. (1988). *EMBO J.* **7**, 589–594.
Johnson, J. D., Pfister, V. R., and Homann, P. H. (1983). *Biochim. Biophys. Acta* **723**, 256–265.
Karlin–Neumann, G. A., Kohorn, B. D., Thornber, J. P., and Tobin, E. M. (1985). *J. Mol. Appl. Genet.* **3**, 45-61.
Kühlbrandt, W., and Wang, D. N. (1991). *Nature (London)* **350**, 130–134.
Laasch, H. (1992). *Z. Naturforsch.* **47c**, 717–725.
Laszlo, J. A., Baker, G. M., and Dilley, R. A. (1984). *Biochim. Biophys. Acta* **764**, 160–169.
Machold, O., Simpson, D. J., and Møller, B. L. (1979). *Carlsberg Res. Commun.* **44**, 235–254.
Nagle, J. F., and Dilley, R. A. (1986). *J. Bioenerg. Biomembr.* **18**, 55–64.
Ouijja, A., Farineau, N., Cantrel, C., and Guillot-Salomon, T. (1988). *Biochim. Biophys. Acta* **932**, 97–106.
Peter, G. F., and Thornber, J. P. (1991). *J. Biol. Chem.* **266**, 16745–16754.
Pfister, V. R., and Homann, P. H. (1986). *Arch. Biochem. Biophys.* **246**, 525–530.
Pfündel, E. E., and Dilley, R. A. (1993). *Plant Physiol.*, **101,** 65–71.
Pick, U., Weiss, M., and Rottenberg, H. (1987). *Biochemistry* **26**, 8295–8302.
Prochaska, L. J., and Dilley, R. A. (1978). *Arch. Biochem. Biophys.* **187**, 61–71.
Prochaska, L. J., and Gross, E. L. (1977). *J. Membr. Biol.* **36**, 13–32.
Renganathan, M., Pfündel, E. E., and Dilley, R. A. (1993). *Biochim. Biophys. Acta* **1142**, 277–292.
Ruban, A. V., Walters, R. G., and Horton, P. (1992). *FEBS Lett.* **309**, 175–179.
Ryie, I. J. (1983). *Eur. J. Biochem.* **131**, 149–155.
Sigalat, C., Haraux, F., de Kouchkovsky, F., Hung, S. P. N., and de Kouchkovsky, Y. (1985). *Biochim. Biophys. Acta* **809**, 403–413.
Simpson, D. J. (1979). *Carlsberg Res. Commun.* **44**, 305–336.
Staehelin, L. A., and Arntzen, C. J. (1983). *J. Cell Biol.* **97**, 1327–1337.
Tandy, N., Bhatnagar, D., Hermodson, M. A., and Dilley, R. A. (1982). *J. Biol. Chem.* **257**, 4301–4307.
Theg, S. M., Johnson, J. D., and Homann, P. H. (1982). *FEBS Lett.* **145**, 25–29.
Theg, S. M., Chiang, G. G., and Dilley, R. A. (1988). *J. Biol. Chem.* **263**, 673–681.
White, M. J., and Green, B. R. (1988). *Photosynth. Res.* **15**, 195–203.