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Calcium-Dependent Interaction of Chlorpromazine with the Chloroplast 8-Kilodalton CF_0 Protein and Calcium Gating of H^+ Fluxes between Thylakoid Membrane Domains and the Lumen†

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ABSTRACT: Earlier work suggested that Ca^{2+} ions in the chloroplast thylakoid lumen interact with thylakoid membrane proteins to produce a proton flux gating structure which functions to regulate the expression of the energy-coupling H^+ gradient between localized and delocalized modes [Chiang, G., & Dilley, R. A. (1987) *Biochemistry 26*, 4911-4916]. In this work, one of the phenothiazine Ca^{2+} antagonists, chlorpromazine, was used as a photoaffinity probe to test for Ca^{2+}-dependent binding of the probe to thylakoid proteins. [^{3}H]Chlorpromazine photoaffinity-labels thylakoid polypeptides of M_r 8K and 6K, with generally much less label occurring in other proteins (some experiments showed labeled proteins at M_r 13K-15K). More label was incorporated in circumstances where it is expected that Ca^{2+} occupies the putative H^+ flux gating site, compared to when the gating site is not occupied by calcium. The photoaffinity labeling of the 8-kDa protein was also influenced by the energization level of the thylakoids (less labeling under H^+ uptake energization). The 8-kDa protein was identified by partial amino acid sequence data as subunit III of the thylakoid CF_0 H^+ channel complex. The partial amino acid sequence of the 6-kDa protein (19 residues were determined with some uncertainties) was compared to data in the GCG sequence analysis data base, and no clear identity to a known sequence was revealed. Neither the exact site of putative Ca^{2+} binding to the CF_0 proteolipid nor the site of covalent attachment of the chlorpromazine to the CF_0 component has been identified. Evidence for gating of energy-linked H^+ fluxes by the hypothesized Ca^{2+}-CF_0 gating site came from the correlation between Ca^{2+}-dependent binding of chlorpromazine to the CF_0 8-kDa protein with inhibition of light-driven H^+ uptake into the lumen but no inhibition of H^+ uptake into sequestered membrane domains. When conditions favored a delocalized $\Delta\tilde{\mu}_{H^+}$ coupling mode, less chlorpromazine was bound to the CF_0 structure, and much larger amounts of H^+ ions were accumulated in the lumen. The data support the hypothesis that Ca^{2+} ions act in concert with the 8-kDa CF_0 protein (and perhaps another protein, the 6-kDa polypeptide?) in a gating mechanism for regulating the expression of the energy-coupling H^+ gradient between localized or delocalized coupling modes.

Recent results have been interpreted as supporting the notion that in thylakoids ATP formation appears to be driven by $\Delta\tilde{\mu}_{H^+}$ gradients that are either delocalized (Davenport & McCarty, 1980; Vinkler et al., 1980; Gräber, 1982; Beard & Dilley, 1986, 1988a; Sigalet et al., 1985; Junge, 1987) or localized (Ort et al., 1986; Graan et al., 1981; Horner & Moudrianakis, 1983, 1986; Sigalet et al., 1985; Beard & Dilley, 1986, 1988a; Pick et al., 1987). The localized coupling hypothesis is still controversial, perhaps partly because it is so difficult to propose models which can account for localized energy-coupling proton gradients. However, the data supporting the localized coupling hypothesis are broadly based, and the search goes on for more critical ways to test the hypothesis [cf. Dilley (1990) for a recent review].

A development that strengthens the localized coupling hypothesis, inasmuch as it introduces discrete, controllable processes, is the recent work suggesting that the energy-coupling modes can be reversibly switched or gated between the localized or delocalized modes under the influence of Ca^{2+} ions (Chiang & Dilley, 1987). High levels of KCl or NaCl (≈100 mM) in the thylakoid storage buffer elicit a delocalized cou-

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pling response (Horner & Moudrianakis, 1986; Sigalet et al., 1985; Beard & Dilley, 1986, 1988a), whereas low salt levels (<30 mM) favor a localized coupling response (Chiang, 1989; Beard & Dilley 1986, 1988a). The effect of KCl was readily reversed by washing away the high-salt-containing buffer and suspending the thylakoids in a buffer having sucrose (or sorbitol) as an equivalent osmoticum (Beard & Dilley, 1988a), suggesting that a reversible switch or gating function was operating. Chiang and Dilley (1987) showed that Ca^{2+} ions in the thylakoid lumen are responsible for the switching between coupling modes and that high concentrations of KCl or NaCl are not necessary per se to elicit the delocalized response, inasmuch as Ca^{2+} chelators which permeate into the lumen are sufficient to induce the delocalized coupling response in low-salt stored thylakoids.

A calcium antagonist of the phenothiazine class (chlorpromazine) was shown to photoaffinity-label the 8-kDa CF_0 subunit most heavily under conditions predicted to have the hypothesized Ca^{2+}-gated H^+ flux channel in the gate-closed configuration (Chiang & Dilley, 1989; Dilley & Chiang, 1990). Chlorpromazine is known to photoaffinity-label calmodulin (Prozialeck et al., 1981) and certain non-calmodulin proteins having high-affinity Ca^{2+} binding sites (Roberts et al., 1986), but only when Ca^{2+} is bound to its specific binding site(s). Here we report additional details concerning the photoaffinity labeling of thylakoid proteins by [^{3}H]chlorpromazine in relation to the functionally-defined Ca^{2+}-dependent gating of H^+ fluxes between localized and delocalized energy coupling modes.

Materials and Methods

Chloroplast Thylakoid Isolation and Chlorophyll and Protein Assays. Chloroplast thylakoids were isolated as described by Ort and Izawa (1973) from 14–21-day-old greenhouse-grown peas. The storage media consisted either of "low salt" resuspension medium containing 200 mM sucrose, 5 mM Hepes, pH 7.5, 2 mM $MgCl_2$, and 0.5 mg/mL bovine serum albumin (BSA) or of a "high salt" medium in which the 200 mM sucrose was replaced by 100 mM KCl and 30 mM sucrose. Those media allow for observation of localized or delocalized energy coupling, respectively (Beard & Dilley, 1986, 1988a). Additional salts and components included in the resuspension media are described in the relevant figures or tables. The chlorophyll concentration in the storage media was 2–4 mg of Chl/mL as determined by the method of Arnon (1949). Protein was measured by the Lowry et al. (1951) method.

Luciferin–Luciferase Detection of Flash-Induced ATP Formation. ATP formation induced by single-turnover flashes was followed by the luciferin–luciferase ATP detection technique using a home-built cuvette as described by Beard and Dilley (1986, 1988b).

For the ATP formation assay, thylakoids were incubated at 10 °C in an assay buffer mixture containing 50 mM Tricine–KOH, pH 8.0, 10 mM sorbitol, 3 mM $MgCl_2$, 1 mM KH_2PO_4, 5 mM DTT, 400 nM valinomycin, 0.1 mM ADP, 5 μM diadenosine pentaphosphate (Sigma), an inhibitor of adenylate kinase, and 0.1 mM methyl viologen (MV). Separate injections of reagents into the cuvette could be made through a syringe needle port. Unless specified otherwise, the assay protocol was as follows: t = 0, thylakoids equivalent to 15–25 μg of Chl/mL were added to 800 μL of reaction mixture; t = 2.5 min, 10 μL of reconstituted luciferin–luciferase reagent prepared from the LKB ATP monitoring kit (Beard & Dilley, 1986, 1988b) was added; t = 3.5 min, ATP formation was initiated by a sequence of 100–150 flashes at 5 Hz. At the end of each assay, the signal was calibrated by addition of standard ATP.

Postillumination ATP formation occurring after a flash train is defined as PIP^+ [1] when ADP and P_i were present during the flash train (Beard et al., 1988). For postillumination ATP formation occurring after a flash train of nonphosphorylating flashes, defined as PIP^-, reaction conditions were as above but without ADP and KH_2PO_4. After an incubation period of 3.5 min, a series of 100 flashes at 5 Hz was initiated. ADP and phosphate were injected into the suspension mixture immediately after the last flash. The resultant (PIP^-) ATP formation was calibrated by standard ATP.

Proton Uptake Assays. Proton uptake was determined from direct recordings of pH changes using a semi-micro combination Orion pH electrode. Thylakoids at 25 μg of Chl/mL were incubated in 50 mM sorbitol, 0.3 mM Tricine (pH 7.5), 15 mM KCl, 3 mM $MgCl_2$, 0.1 mM MV, and 400 nM valinomycin in a reaction chamber thermostated at 10 °C.

[^{3}H]Chlorpromazine Photoaffinity Labeling. Labeling of thylakoid proteins with [^{3}H]chlorpromazine (NEN Co., ^{3}H labeling in the benzene ring) was carried out by a modified method of Prozialeck et al. (1981). Thylakoids were prepared either in the low-salt media or in the high-salt media (sometimes with the inclusion of 1.5 mM $CaCl_2$). The addition of $CaCl_2$ was intended to give maximal occupancy of Ca^{2+} binding sites. Thylakoids were diluted with either of the above media, but without BSA, to 2.5 mg of Chl/mL, and a 0.20-mL volume was incubated 30 min on ice with 5 μM [^{3}H]chlorpromazine (4000 mCi/mmol). The thylakoid suspensions were then diluted to 33 μg of Chl/mL with the appropriate buffer, [^{3}H]chlorpromazine (4000 mCi/mmol) was added to a final concentration of 5 μM, and UV illumination was begun and vigorous stirring was maintained. For Figures 1B and 2 and Table II, photoaffinity labeling was induced by a 1-h exposure to two Mineralite broad-band mercury UV light sources (the main emission band being in the UV-C region at 254 nm) positioned at 4 cm over the stirred suspensions. Some experiments used a Universal (Gelman-Camag, made in Switzerland) Model 51402 UV-C (main band at 254 mm) source. The experiments of Tables III–V used a 20-min UV exposure. The chloroplasts were recovered by sedimentation at 20000g for 10 min and then washed 3 times in 5 mM Hepes, pH 7.5, 0.5 mM $MgCl_2$, and 1 mM unlabeled chlorpromazine.

For preparing samples for SDS–PAGE gels, the thylakoids were delipidated by three washes with 90% acetone and solubilized in 10% SDS. In addition to aiding the running of SDS–PAGE gels, the washes removed any noncovalently associated labeled chlorpromazine. The protease inhibitors phenylmethanesulfonyl fluoride (PMSF), benzamidine, and ε-amino-*n*-caproic acid were included in all solutions used to prepare the proteins for SDS–PAGE.

The [^{3}H]chlorpromazine labeling protocol for thylakoids from which the DCCD-sensitive 8-kDa CF_0 subunit was to be purified (see below) was essentially the same, except that thylakoids with 4 mg of Chl were treated with 5 μM [^{3}H]-chlorpromazine at 1500–2000 mCi/mmol.

[^{14}C]Dicyclohexylcarbodiimide Labeling. The [^{14}C]DCCD label was incorporated into proteins according to Nelson et al. (1977). For single-label experiments, low-salt-stored thylakoids at 2.5 mg of Chl/mL (4 mg of Chl total) were

[1] Abbreviations: CPZ, chlorpromazine; PIP^+, postillumination phosphorylation (ATP yield) under conditions of having ADP and P_i present during the illumination; PIP^-, postillumination phosphorylation, where ADP and P_i are added after the illumination; Pyr, pyridine; TF, trifluoperazine.

stirred for 1 h at 25 °C in a medium consisting of 0.2 M sucrose, 5 mM Hepes–NaOH, pH 7.5, and 2 mM $MgCl_2$ in the presence of [^{14}C]DCCD (specific activity 5 mCi/mmol, 33 nmol/mg of protein). After the labeling treatment, the membranes were centrifuged for 10 min at 20000*g* and washed twice in the same medium (without DCCD) followed by the above-mentioned 90% acetone washes.

For the double-labeled experiments, [^{3}H]chlorpromazine-labeled thylakoids were centrifuged at 2000*g* for 10 min and washed twice before being stirred at 2.5 mg of Chl/mL in the [^{14}C]DCCD reaction buffer described above.

Isolation of the 8-kDa CF_0 Subunit. The DCCD-sensitive CF_0 proteolipid was extracted from nonlabeled and [^{3}H]-chlorpromazine (and/or [^{14}C]DCCD)-radiolabeled thylakoids according to the procedure of Nelson et al. (1977). Chloroplasts equivalent to 4 mg of Chl in 1 mL of H_2O were injected into 50 mL of cold 1-butanol (3–4 °C) and stirred vigorously for 45 min. The precipitated material was removed by filtering 3 times through Whatman no. 3 paper and the filtrate added, with stirring, to 250 mL of diethyl ether precooled to −20 °C. After 2.5 h, the precipitate was collected at −20 °C by centrifugation for 20 min at 10000*g*. The recovered protein was washed in cold 1-butanol and solubilized in 3–4 mL of $CHCl_3$/MeOH (2:1). The protein was obtained by drying under nitrogen, and samples were solubilized in 10% SDS for SDS–PAGE.

Reconstitution of Purified CF_0–CF_1 into Azolectin Vesicles. The purified nine-subunit CF_0–CF_1 complex, isolated as in Schmidt and Gräber (1985) (kindly supplied by Prof. Peter (Gräber), was reconstituted into azolectin vesicles following the procedures of Schmidt and Gräber (1985).

SDS–Polyacrylamide Gel Electrophoresis and Assays for Radioactivity. Electrophoresis of proteins solubilized in 10% SDS was carried out by a modified method by Laemmli (1970). The gels were scanned for stained (Coomassie blue) protein at 580 nm with an ISCO gel scanner (Model 1312). The radiolabel associated with each peak was determined either by cutting the gel into 2-mm segments or by excising the entire visible stained bands. The slices were decolorized in 0.6 mL of 30% H_2O_2 in tightly capped scintillation vials at 80 °C for 4 h. After the vials were cooled, 10 mL of aqueous counting scintillant (Research Products International, Inc.) was added. Chemiluminescence was allowed to decay in the dark for 7 h before samples were counted in a Searle Isocap 300 scintillation counter. For dual-labeled experiments, the samples were counted separately for ^{3}H and ^{14}C. Overlap of the ^{14}C counts into the ^{3}H window was determined by adding 5 μL of ^{14}C standard (2.5×10^5 dpm/mL) to each vial and recounting in the ^{14}C and ^{3}H channels.

Reversed-Phase High-Performance Chromatography. Isolation of the DCCD-sensitive proteolipid according to the procedure of Nelson et al. (1977) yielded two major proteins of M_r 8K and 6K, both for chlorpromazine-treated and for control thylakoids. Protease inhibitors were used in the isolation procedures, so the 6-kDa protein should not have been a proteolysis product. Further purification of these proteins was accomplished on a poly(styrenedivinylbenzene) column (Tweeten & Tweeten, 1986). A total of approximately 1 mg of protein was accumulated for four proteolipid preparations. Each preparation involved extraction of thylakoids equivalent to 8 mg of Chl, as described above.

Protein samples (200–400 μg of Chl) were solubilized in 80% formic acid (Aldrich) before injection onto the column. Reverse-phase chromatography was performed on a Varian Model 5500 gradient pumping system equipped with a Valco Model C6U injection valve with a 100-μL sample loop with a Perkin-Elmer LC 85B variable-wavelength UV detector operated at 280 nm. Separations were achieved on a poly(styrenedivinylbenzene) PLRP-S matrix (Polymer Laboratories, Shropshire, U.K.) of pore diameter 1000 Å and particle size 8 μm packed in a 5 × 0.41 cm (i.d.) column. Protein elution was monitored at 280 nm and accomplished with formic acid/acetonitrile linear gradients at a flow rate of 1 mL/min.

Samples of each peak were analyzed by mini-gel SDS–PAGE to identify fractions containing the 8- and 6-kDa proteins.

Amino Acid Sequence Analysis. The 8- and 6-kDa proteolipids separated by HPLC were deblocked at the N-terminus by incubation in methanolic 0.5 N HCl for 24 h at room temperature. The amino acid sequencing of the first 5 amino acids of the 8-kDa protein and first 20 amino acids of the 6-kDa protein was carried out by Prof. M. Hermodson and Ms. Mary Woerner (Purdue University Biochemistry Department) on the Applied Biosystems Model 470A gas-phase protein/peptide sequencer.

Results

Photoaffinity Labeling of Thylakoid Proteins with [^{3}H]-Chlorpromazine. The principle behind the experiments reported here is that thylakoids were exposed to [^{3}H]chlorpromazine and UV light under conditions, indicated from our functional studies (Chiang & Dilley, 1987), wherein Ca^{2+} ions are either (a) bound to a putative H^+ flux gating site (giving a localized energy coupling response) or (b) displaced from the gating site (giving a delocalized coupling response). Phenothiazine drugs such as chlorpromazine (CPZ) or trifluoperazine (TF) are known to bind tightly to the Ca^{2+} bound form of calmodulin (Klee et al., 1980) as well as to non-calmodulin Ca^{2+} binding proteins (Roberts et al., 1986). The Ca^{2+}-dependent protein–chlorpromazine complex becomes covalently linked after UV excitation of the CPZ (Prozialeck et al., 1981).

As we reported earlier, thylakoids exposed to UV photoactivation in the presence of 5 μM [^{3}H]CPZ resulted in labeling of several polypeptide bands in the 6–15-kDa region [cf. Figure 2 of Chiang and Dilley (1989) or Figure 7 of Dilley and Chiang (1989)]. Nearly twice as much label was incorporated into the proteins isolated from low-salt-stored thylakoids compared to those from the high-salt stored case, i.e., more labeling under conditions predicted to have Ca^{2+} occupying the putative H^+ flux gating site. Particularly prominent and very reproducibly appearing labeled bands in the SDS–PAGE gels ran with apparent molecular masses near 8 and 6 kDa. The labeled bands in the 13–15-kDa region were not as reproducibly observed. The possibility that the 8-kDa band in question was the 8-kDa CF_0 subunit (subunit III) was tested by isolating subunit III using the Nelson et al. (1977) butanol extraction/ether precipitation procedure. In our hands, that preparation gives two prominent bands, the expected 8-kDa band and a 6-kDa band of less stain intensity (Dilley & Chiang, 1990). Figure 1A shows densitometry profiles of typical SDS–PAGE gels of control thylakoids having neither CPZ nor UV treatments, and both bands were present. Figure 1B shows similar protein isolation after UV treatment of thylakoids in the presence of [^{3}H]CPZ [previously published (Chiang & Dilley, 1989) but reproduced here for clarity]. The 6-kDa band was not reported in the original work by Nelson et al. (1977), but was observed by Tandy et al. (1982). For the data shown in Figure 1B, low-salt-stored thylakoids were given 1-h UV treatment in the presence of 5 μM [^{3}H]CPZ,

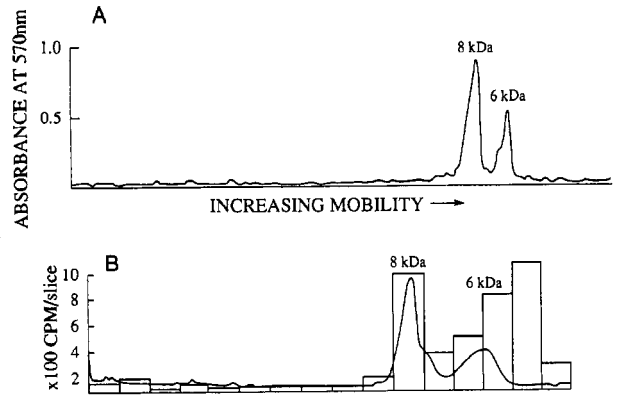

FIGURE 1: (A) Optical density profile of an SDS–PAGE gel of an 8-kDa CF_0 preparation treated in the absence of chloropromazine or UV light. The CF_0 protein was purified, and SDS–PAGE gels were run as described under Materials and Methods, from low-salt-stored thylakoids. Samples were loaded on 15% SDS–PAGE gels at ≈50 μg of protein/lane. (B) Optical density profile and photoaffinity [^{3}H]chlorpromazine labeling pattern of an 8-kDa CF_0 preparation separated by SDS–PAGE. Thylakoids (low-salt-stored) were photoaffinity-labeled at 33 μg of Chl/mL with 5 μM [^{3}H]CPZ for 1 h in UV light, followed by butanol extraction and ether precipitation to obtain the 8-kDa CF_0 protein (see Materials and Methods). Samples were loaded on 15% SDS–PAGE gels at ≈50 μg of protein/lane. Radioactivity was counted in 5-mm gel slices, and the counts are given by the bar graph. The data are the average of three gel lanes.

Table I: Partial Amino Acid Sequence of the 8- and 6-kDa Proteins Separated by Reverse-Phase HPLC[a]

8-kDa CF_0 (literature)[b]	f-Met-Asn-Pro-Leu-Ile^5-Ala-Ala-Ala-Ser-Val^{10}-Ile-Ala-
8 kDa	f-Met-Asn-Pro-Leu-Ile
6 kDa[c]	Pro-(Ser,Ala)-Leu-His-Leu-?Lys^5-?-?Ser-Val-Ala-Pro^{10}-Val-Ala-?Trp-?Ile-?Phe^{15}-?Ile-?Asp?-Phe-?Asp

[a] Procedures used were as described under Materials and Methods. [b] The published amino acid sequence of the 8-kDa CF_0 subunit is for pea chloroplast DNA (Huttley & Gray, 1984). [c] The uncertainties in the assignments of some residues are indicated; the first residue could have been Ser or Ala; the fifth residue assignment as Lys is not as firm as one should expect, and a similar uncertainty exists for Ser (7) and the residues from Trp (13) on. The sixth residue could not be assigned.

followed by the butanol/ether isolation procedures and SDS–PAGE of the ether-precipitated pellet. Both an 8-kDa band (expected to be CF_0 subunit III) and the 6-kDa band were labeled with an additional strong peak of label running ahead of the 6-kDa band.

To test whether the 6-kDa band is an altered form of subunit III, both the 8- and 6-kDa bands were purified further, starting with the butanol/ether preparation, by reversed-phase HPLC employing a new type of large-pore poly(styrenedivinylbenzene) column (Tweeten & Tweeten, 1986). Fractions from the column were analyzed by SDS–PAGE minigels, showing that the 6-kDa band eluted at 22 min and the 8-kDa band at 32 min. Pooled fractions from three HPLC runs were subjected to a methanolic 0.5 N HCl deblocking procedure (see Materials and Methods). The 8-kDa CF_0 is known to have a formyl group on the N-terminal methionine (Sebald & Hoppe, 1981; Sebald & Wachter, 1980), but we were not sure whether the 6-kDa protein was formylated—the deblocking step was thus considered a reasonable procedure. Partial amino acid sequencing was carried out (courtesy of Prof. M. Hermodson and Ms. Mary Woerner, Purdue University), and the results (Table I) clearly showed the following: (1) the 8-kDa band is indeed the CF_0 subunit, shown by comparison with the sequence reported in the literature (Huttley & Gray, 1984); (2) the 6-kDa polypeptide does not correspond to any part of the 8-kDa CF_0 subunit. Computer searches of available sequence data banks were made by Mr. Rick Westerman (Purdue University Biochemistry Department) using the GCG sequence analysis program. Using the 17 amino acids starting from position 2 (Leu), the fitting program indicated 58% similarity and 31% identity to an ATP synthase gene (ATP 1) of *Anabaena* sp. (McCarn et al., 1988). That gene is closely related to the unc 1 gene of *Escherichia coli*, the function of which has not been discovered, nor has the predicted amino acid been observed in SDS–PAGE gels. This raises obvious and interesting possibilities, presently being investigated. A 6-kDa band was also observed in the SDS–PAGE gels of the purified CF_0–CF_1 provided by P. Gräber (Figure 3) (see below for further comment).

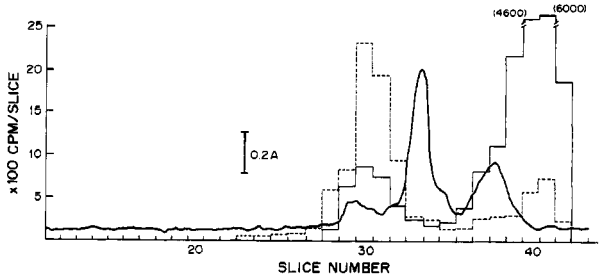

FIGURE 2: Optical density profile and dual-labeling pattern for [^{14}C]DCCD and [^{3}H]chlorpromazine associated with an 8-kDa CF_0 preparation separated by SDS–PAGE. The CPZ concentration and UV light treatment were as in Figure 1B (for other details, see Materials and Methods), and the isolation procedure was the butanol extraction and ether precipitation method with SDS–PAGE gels to obtain the 8-kDa CF_0 protein. Samples were loaded on 15% SDS–PAGE gels at ≈50 mg of protein/lane (cf. Materials and Methods). The heavy line is the Coomassie blue stain density; [^{3}H]CPZ (—); [^{14}C]DCCD (---). The numbers in parentheses indicate the radioactivity (CPM) of the two off-scale fractions.

Additional experiments to test for [^{3}H]CPZ labeling of the 8-kDa CF_0 subunit were carried out using a dual-labeling approach with [^{14}C]DCCD and [^{3}H]CPZ. Low-salt-stored thylakoids were UV-irradiated in the presence of 6 μM [^{3}H]CPZ, washed by centrifugation, and resuspended in fresh medium with 50 μM [^{14}C]DCCD for a 1-h treatment. Preparation of the butanol/ether protein fraction was carried out followed by SDS–PAGE analysis of the gels by slicing into 2-mm segments and counting the fractions by scintillation counting (see Materials and Methods). The results of that experiment (Figure 2) show that both [^{14}C]DCCD (dashed line) and [^{3}H]CPZ (solid line) were present in the same fractions (28–32), which coincide with the position of a smaller stained band compared to the main band in fractions 33 and 34. The minor, higher molecular mass band associated with the 8-kDa band only appears after DCCD treatment of the thylakoids (note the absence of the minor band in Figure 1B and a barely detectable shoulder in that region in Figure 1A) as shown in several earlier studies of the DCCD-derivatized protein (Nelson et al., 1977; Doherty & Gray, 1980; Tandy et al., 1982, 1983; Hermolin & Fillingame, 1989). Labeling the 8-kDa subunit with only [^{3}H]CPZ resulted in no such shift in molecular mass, as shown by the data in Figure 1B. This difference between CPZ and DCCD is perhaps to be understood as owing to the fact that DCCD reduces, by one, the negative charge of the protein [reacting with Glu-61 (Sebald & Wachter, 1980)], whereas CPZ may not change the net charge. The facts (A) that in CPZ-labeled material [^{3}H]CPZ runs with the main 8-kDa band (Figure 1B) and in double-labeled membranes [^{3}H]CPZ runs with the [^{14}C]DCCD-containing minor band, (B) that it is expected that 50 μM

DCCD treatment for the relatively short time used in these experiments would only label a small proportion of the 8-kDa CF_0 in thylakoids (Nelson et al., 1977), and (C) that there is no other DCCD-labeled material (or protein) in the butanol/ether method preparation which runs with a slightly higher molecular mass, other than the CF_0 with an altered molecular mass (cf. the references above validating this point), provide sound evidence for the 8-kDa CF_0 being a target for CPZ labeling. The interesting implication of the dual-labeling pattern in Figure 2 is that CPZ labeling occurred only (or mainly) on the fraction of the 8-kDa CF_0 that was predisposed to be most reactive with DCCD. That is, there may be some heterogeneity in the population of the 8-kDa CF_0, with a portion which is reactive with both probes in the time frame of our experiments and a portion that is less reactive.

The relationship between the 6-kDa protein and CPZ probe binding is not so clear, because the [^{3}H]CPZ label is highest in the gel region below the main 6-kDa band (Figures 1B and 2), although there is a significant amount of label coincident with the stained band. Interestingly, there is a noticeable amount of [^{14}C]DCCD in fractions 40 and 41 of Figure 2, which also have the greatest amount of [^{3}H]CPZ label. It is not likely that the probes present in fractions 40 and 41 are free or in micellar form, because both probes are very soluble in acetone and the labeled thylakoids were given three acetone washes prior to solubilization in SDS. The acetone washes would probably have removed any free or micellar forms of either probe. It seems more likely that the probes are covalently linked to proteins which run at the position of fractions 40 and 41, but either the proteins do not stain well with Coomassie blue or there is a high level of labeling associated with a small amount of the protein which normally runs ahead of the 6-kDa band. The dye front in gels as those used for the data in Figures 1B and 2 runs close to 1 cm below the 6-kDa stained band, considerably below the position of the radioactivity peaks.

The purified CF_0–CF_1 complex (provided by Prof. Peter Gräber), reconstituted into liposomes, also showed [^{3}H]CPZ photoaffinity labeling. The reconstituted vesicle preparation was exposed to UV radiation in the presence of [^{3}H]CPZ followed by SDS–PAGE. Figure 3 shows the radioactivity counts of slices cut from the gel superimposed on a densitometry scan of the Coomassie blue stain. The five CF_1 subunits (α–ϵ) of CF_1 were clearly observed by staining, but they were not labeled by [^{3}H]CPZ. The identity of the subunits was assigned according to their known molecular masses and the positions of the molecular weight standards used in the gel. As noted by the identifying symbols below the gel, we presume that the ϵ of CF_1 and subunit II of CF_0 are in one band running near 15 kDa and subunits I and IV are in a band just below the δ band of CF_1 near 19 kDa. The CF_0 subunits I, II, and possibly IV were not labeled, whereas the 8-kDa CF_0 subunit III was heavily labeled. A stained band near 6 kDa was also labeled, but less than the 8-kDa band. The heaviest labeling occurred in a region on the low molecular mass shoulder of the 6-kDa band, consistent with data of Figures 1B and 2.

Ca^{2+} Effects on Chlorpromazine Labeling of the 8-kDa CF_0 Protein. Previous work resulted in our suggesting that low-salt-stored thylakoids carry out energy coupling utilizing H^+ gradients constrained to some type of localized domains whereas treatments such as storage in a high KCl or NaCl medium (Beard & Dilley, 1988a) or having Ca^{2+} chelators present in the low-salt storage medium (Chiang & Dilley, 1987) resulted in a delocalized energy coupling mode. That Ca^{2+} ions present in the lumen (or bound to the lumenal side

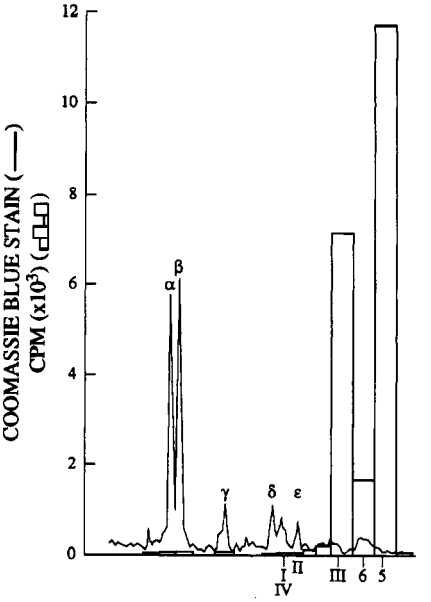

FIGURE 3: [^{3}H]CPZ labeling of purified CF_0–CF_1 reconstituted into azolectin vesicles. The purified CF_0–CF_1 (kindly provided by Dr. P. Gräber) was reconstituted and [^{3}H]CPZ UV photoaffinity labeling treatment was given as described under Materials and Methods. The CF_0–CF_1 proteins were then isolated on SDS–PAGE gels, and the stained bands (solid line from a densitometer scan) were cut out and counted for radioactivity (given by the bar graph data). The five CF_1 subunits (α–ϵ) are identified by their respective symbols. The CF_0 subunits are identified by Roman numerals; subunits I (19 000 kDa) and IV (19 000 kDa) share one band, subunit II (15 000 kDa) runs together with the CF_1 ϵ subunit, and subunit III (8000 kDa) is in a broad band. The stain pattern indicates that a 6-kDa polypeptide was present. The molecular masses expected for the CF_0–CF_1 subunits were consistent with molecular mass standards (Pharmacia) run on the same gel (albumin, 66 kDa; ovalbumin, 45 kDa; chymotrypsin, 25 kDa; myoglobin I, 17.2 kDa; ribonuclease A, 13.7 kDa; myoglobin II, 6.38 kDa). The numbers 6 and 5 on the lower right and the vertical marks above them indicate the predicted location for 6- and 5-kDa molecular masses, respectively.

of the membrane) are the key factor regulating the two different coupling responses was indicated by the observation that the shift between localized or delocalized coupling modes is reversible by the appropriate control of Ca^{2+} levels. For example, if the high-KCl medium was supplemented by 1 mM $CaCl_2$, or if 5 mM $CaCl_2$ were added to the 2 mM EGTA chelator treatment, the thylakoids showed a localized energy coupling response (Chiang & Dilley, 1987). Other divalent cations tested (Mn^{2+} and Mg^{2+}) did not replace the Ca^{2+} effect; however, $LaCl_3$ was effective at 0.1 mM La^{3+} (unpublished data). The latter observation implies that this Ca^{2+} interaction site belongs to the general class of biologically broadly distributed, high-affinity Ca^{2+} binding sites because La^{3+} is known to replace Ca^{2+} in many proteins having Ca^{2+}-specific binding sites (Synder et al., 1990).

It is well-known that calmodulun antagonists such as chlorpromazine bind tightly to the protein only when Ca^{2+} (or La^{3+}) is present in the specific cation binding sites. Moreover, such drug binding is observed for other, non-calmodulin proteins having high-affinity Ca^{2+} binding sites (Klee et al., 1980; Roberts et al., 1986). Therefore, it was of interest to characterize the Ca^{2+} effects on CPZ binding to the thylakoid proteins identified in this work, even though they are not likely to be of the calmodulin group (calmodulin has not been found in thylakoids; Simon et al., 1984).

Table II indicates that CPZ gave greater photoaffinity labeling of the 6- and 8-kDa proteins stored and UV-treated under the low-salt conditions compared to thylakoids under the high-salt conditions. The low-salt conditions are precisely

Table II: Chlorpromazine Photoaffinity Labeling of the 6- and 8-kDa Proteins Purified from Low-Salt- or High-Salt-Stored Thylakoids by the Butanol/Ether Method[a]

thylakoid storage conditions	chlorpromazine binding (cpm/unit area of protein stain) 8 kDa	6 kDa
low salt	196 ± 8	451 ± 50
high salt	121 ± 4	230 ± 5

[a] Procedures for [^{3}H]CPZ labeling by UV photoaffinity activation were as given under Materials and Methods. The UV exposure time was 1 h at ice bath temperature. The 6- and 8-kDa polypeptides were isolated by the butanol extraction/ether precipitation method and purified by SDS–PAGE (see Materials and Methods), and 25 μg of total protein was loaded per gel lane. The resulting stained bands were cut out of the gels and counted for radioactivity. The data are averages ± the standard deviation of three separate lanes loaded with 25 μg of protein each, and each sample was counted 3 times. On the basis of the specific activity of [^{3}H]CPZ and the recovered counts, the labeling is calculated as approximately 1 mol of CPZ/10^5 mol of CF_0 complex or 1 mol of CPZ/10^4 mol of 8-kDa polypeptide. The specific activity of the [^{3}H]CPZ used in the labeling reaction was 5.3×10^9 cpm/μmol. The low level of labeling has been noted in other work using [^{3}H]CPZ as a photoaffinity probe in non-calmodulin proteins (Oswald & Changeux, 1981).

those predicted from our functional assays to have Ca^{2+} binding to the putative H^+ flux gating site. A further test of the notion that Ca^{2+} is involved in CPZ tight binding (leading to a higher level of photoaffinity labeling) was to add Ca^{2+} to the high-KCl storage medium prior to UV treatment. In the ATP formation functional assays which show either localized or delocalized energy coupling, adding 0.5–1.0 mM $CaCl_2$ to the high-KCl thylakoid storage medium caused a virtually complete blocking of the high-KCl effect of inducing the delocalized energy coupling response, and those thylakoids gave the same localized coupling response as low-salt-stored membranes (Chiang & Dilley, 1987). Thus, it was expected that an increase in [^{3}H]CPZ binding would occur in the high-salt-stored sample supplemented with $CaCl_2$. Table III shows that storing and UV treating thylakoids in the high-salt (100 mM KCl) medium supplemented with 1 mM $CaCl_2$ resulted in more than a doubling of the CPZ photoaffinity labeling of the 8-kDa CF_0 protein compared to thylakoids stored and treated in the regular high-salt medium. In that experiment, the low-salt-stored sample (plus 1 mM $CaCl_2$) had more CPZ bound than the high-salt case (without $CaCl_2$ added), but not as much as when the high-salt thylakoids were supplemented with 1 mM $CaCl_2$. The fact that the low-salt storage treatment (in this case with 1 mM $CaCl_2$ added) did not give as much labeling as the high-salt-stored (+1 mM $CaCl_2$) sample indicates that there are probably additional factors at play, not fully understood, which govern the photoaffinity labeling level. However, the general response, over many repeated experiments, was that the high-salt-stored samples had significantly less CPZ label than the low-salt-stored sample and Ca^{2+} added to the high-salt-stored case greatly increased the [^{3}H]CPZ labeling level. One factor, discussed next, that greatly influences CPZ photoaffinity labeling is the energization (by H^+ uptake) of the membrane.

Effect of Membrane Energization on CPZ Labeling. The UV-C (enriched) light sources we used, Universal (Gelman-Camag) Model 51402 and Mineral Light (UVP, Inc., Model UVGL-25), have a low, but significant, level of blue radiation adsorbed by chlorophyll which energizes electron transport and H^+ uptake. Typical H^+ uptake measurements (under similar conditions as given in Figure 5) with a 5-mL cuvette having 20 μg of Chl mL^{-1} and irradiated from the side with the

Table III: Effect of Adding $CaCl_2$ to the High-Salt Storage Medium on CPZ Photoaffinity Labeling of the 8-kDa Protein[a]

thylakoid storage conditions	cpm in gel slice	nmol of protein in gel slice	chlorpromazine content (CPZ/protein × 10^{-5})
high salt	83 ± 5[b]	2.0 ± 0.3[c]	2.2 ± 0.3[d]
high salt plus 1 mM $CaCl_2$	211 ± 11	1.9 ± 0.1	5.9 ± 0.6
low salt plus 1 mM $CaCl_2$	74 ± 3	1.3 ± 0.1	3.2 ± 0.3

[a] Procedures used were similar to those given in Table II (UV exposure was for 20 min) utilizing the butanol/ether and SDS–PAGE purification method to isolate the 8-kDa protein. The [^{3}H]CPZ specific activity was 1.86×10^9 cpm/μmol. The Coomassie-stained bands corresponding to the 8-kDa polypeptide were cut out of the SDS–PAGE gel and analyzed for radioactivity after obtaining a densitometer trace of the gel. The proportion of stain density of the 8-kDa band compared to the total stain density made up of the 8- and 6-kDa proteins was used to calculate the proportion of the 25 μg of total protein loaded that was assumed to be in the 8-kDa band. That amount of protein was used to estimate the nanomoles of the 8-kDa protein in the gel band. This may be only a rough approximation owing to our assumption that the two proteins take up stain in a similar way. While this may not be exact, it is suitable for purposes of comparison of each protein between the different samples. Background counts were subtracted in all cases (≈25 cpm). In this experiment, 35 μg of protein was added to each of three lanes for each of the three treatments. The samples were counted 3 times for radioactivity prior to spiking representative vials with a known amount of ^{3}H counts to check for any variation in quenching (no variation was found between samples). [b] The standard deviations listed represent the data from the total of nine observations of the counts from each treatment. [c] The standard deviations for this parameter were calculated from the counts of three separate gel slices. [d] The standard deviation for this parameter was calculated from the three values obtained using the data from the second column (expressed as nanomoles of CPZ per band) and the third-column values.

Universal UV-C source gave 42 ± 4 nmol of H^+ uptake (mg of Chl)$^{-1}$ with no methyl viologen added and 41 ± 9 nmol of H^+ (mg of Chl)$^{-1}$ in the presence of methyl viologen (oxygen apparently acted as a PSI acceptor in the absence of methyl viologen). Addition of 1 μM nigericin completely blocked H^+ uptake, indicating that the pH changes observed were due to H^+ transport activity. The Mineralite UV-C lamp gave 82 ± 7 nmol of H^+ (mg of Chl)$^{-1}$ with methyl viologen and 57 ± 11 nmol of H^+ (mg of Chl)$^{-1}$ without methyl viologen, and for both conditions, the presence of 5 μM nigericin completely blocked H^+ uptake.

Experiments were carried out to test for the effect of the low level of energization on [^{3}H]CPZ labeling. As Table IV (A and B) shows, inhibiting electron transport with 20 μM DCMU (H^+ uptake would also be inhibited) or inhibiting H^+ uptake, but not electron transport, with the uncoupler combination of 5 μM nigericin plus 1 μM nonactin gave roughly a 2-fold increase in [^{3}H]CPZ label incorporation into the butanol/ether precipitate containing the 8-kDa proteolipid and the 6-kDa polypeptide. Either low- or high-salt-stored thylakoids gave the same effect, although the higher labeling level of the latter is explained by the experiment being done on a different day than the low-salt-stored sample and using a higher specific activity of [^{3}H]CPZ. The higher labeling level of the proteolipids under deenergized conditions suggests the possibility that the energization may have its effect through H^+ accumulation causing the displacement of Ca^{2+} from a binding site and consequently lower CPZ binding to the protein(s). To test that notion, a Ca^{2+} ionophore (5 μM A23187) and 2 mM EGTA as an externally-located Ca^{2+} trap were added to a combined DCMU + nigericin treatment (experiment C of Table IV). The external Ca^{2+} trap caused the otherwise high [^{3}H]CPZ labeling level of the deenergized treatment to be decreased to near that of the energized sample.

Table IV: Effect of DCMU and Nigericin on [^{3}H]CPZ Labeling of the Thylakoid 8- and 6-kDa Proteolipids

thylakoid treatment	cpm/μg of protein
(A) Low-Salt-Stored Thylakoids[a]	
(1) control	92 ± 5
(2) +20 μM DCMU	253 ± 6
(3) +5 μM nigericin	267 ± 6
(B) High-Salt-Stored Thylakoids[b]	
(1) control	561 ± 60
(2) +20 μM DCMU	1204 ± 30
(3) +5 μM nigericin	702 ± 21
(C) Low-Salt-Stored Thylakoids[c]	
(1) control	690 (27)
(2) +20 μM DCMU + 5 μM nigericin	1330 (149)
(3) as (2) + 5 μM A23187 + 2 mM EGTA	733 (34)
(C) High-Salt-Stored Thylakoids	
(1) control	433 (4)
(2) +20 μM DCMU + 5 μM nigericin	700 (96)
(3) as (2) + 5 μM A23187 + 2 mM EGTA	588 (325)

[a] Experiment A. Procedures were similar to those described under Materials and Methods and in Table III for thylakoid treatment and UV exposure. Low-salt-stored thylakoids were used; DCMU and nigericin were added as indicated prior to the 20-min UV treatment. Nonactin (1 μM) was present in all the treatments. The 8- and 6-kDa thylakoid proteins were isolated using the butanol/ether procedure (see materials and Methods and Figure 1), and aliquots of the SDS-solubilized ether precipitate (containing both proteins) were counted for radioactivity. Three separate thylakoid samples were given each treatment, and a sample of each was counted 3 times. [b] Experiment B. Similar procedures were used as in experiment A for butanol/ether SDS–PAGE isolation of the 8-kDa proteolipid. High-salt-stored thylakoids were used. The different labeling level compared to experiment A is accounted for by the experiment being carried out on a different day with a higher specific activity of [^{3}H]CPZ being used. [c] Experiment C. In this experiment, whole thylakoid proteins, either low- or high-salt-stored, were run on SDS–PAGE gels after the [^{3}H]-CPZ treatments, and only the 8-kDa band was cut out and counted. Similar conditions were used as for parts A and B except that both low- and high-salt-stored thylakoids were used, and DCMU and nigericin were combined (in row 2). The treatment in row 3 also had DCMU and nigericin as row 2, but in addition, an external "Ca^{2+} trap" (2 mM EGTA) was present and 5 μM A23187 to allow fast equilibration of Ca^{2+} across the thylakoid. The 8-kDa band was cut out of two SDS–PAGE gels and prepared for scintillation counting. The values listed are the averages of the two samples, and the ranges are given in parentheses as counts above or below the average value.

Hence, it appears that energization could cause a displacement of Ca^{2+} from the putative CF_0 binding site or induce protonation-dependent structural changes in the CF_0, either of which could, in principle, result in lower [^{3}H]CPZ binding.

Recalling that the conditions of low-salt storage or 1 mM $CaCl_2$ present in the high-salt storage were those under which the phosphorylation assays reported earlier gave a localized energy coupling pattern (Beard & Dilley, 1988a; Chiang & Dilley, 1987), we are logically led to the notion that Ca^{2+} ions, bound at the putative 8-kDa CF_0 binding site, act as a gate to keep H^+ ions constrained to a localized phase. Displacing the Ca^{2+} is postulated to cause the gate being open, allowing H^+ ions to equilibrate with the lumen phase, giving the observed delocalized coupling response. The suggestion that Ca^{2+} ions are involved in a type of gating action of H^+ fluxes can be tested further by direct measurement of H^+ uptake in an experiment wherein we can distinguish between H^+ ions taken up in the localized domains from those accumulated in the lumen. We have shown previously that is is possible to distinguish H^+ uptake into the lumen from H^+ uptake into the sequestered domains by using amine buffers such as pyridine (pK_a 5.44) or (hydroxyethyl)morpholine (pK_a 6.2) (Beard et al., 1988; Renganathan et al., 1991). The amines freely equilibrate with the lumen, and when lumenal H^+ uptake causes the internal pH to drop into the buffering range of the amine, an additional H^+ uptake component is observed owing to the amine buffering action (Nelson et al., 1971; Avron, 1972). The data in Renganathan et al. (1991) (Figure 3) can be consulted for an example of the markedly different response of thylakoid H^+ uptake to the two amines when given to thylakoids predicted to be expressing either localized or delocalized H^+ gradients. Phenothiazine drugs are known from other work to increase Ca^{2+} binding affinity to the specific binding sites in Ca^{2+} binding proteins (Andersson et al., 1983), so this provides an additional tool for testing the hypothesis concerning the gating action on H^+ fluxes.

Chlorpromazine Effects on H^+ Transport and Phosphorylation. It was necessary before proceeding further with this approach to know what effects the phenothiazine drugs have on energy coupling parameters under the conditions we use. Previous work has shown that CPZ in the 5–20 μM range inhibits ATP formation *when present in the phosphorylation assay* (Good et al., 1966). In the experimental protocols reported here, high concentrations of phenothiazines were avoided in the final phosphorylation or H^+ uptake assay medium by giving the drug (up to 10 μM) *only* during the thylakoid storage stage. A typical 100–150-fold dilution of the stock thylakoids into the assay medium results in a final concentration of phenothiazine of less than 0.05 μM.

Effects of Phenothiazines on ATP Formation. Using that protocol, the low final concentrations of CPZ or TF did not inhibit flash-induced ATP formation (Table V). Addition of a 4 μM aliquot of either drug to the storage treatments for low-salt (rows 2 and 3, Table V) or high-salt-stored thylakoids (row 5) caused no discernible effect on the ATP yield per flash

Table V: Effect on Chlorpromazine and Trifluoperazine, Present Only in the Thylakoid Storage Stage, on the Onset of ATP Formation and ATP Yield per Flash[a]

thylakoid storage condition	energization lag, no. of flashes			yield/flash (nmol of ATP/mg of Chl)		
	−Pyr	+Pyr	+Pyr − −Pyr	−Pyr	+Pyr	energy coupling mode
(1) control (low salt)	34/39	36/42	2/3	0.49	0.46	localized
(2) low salt, 4 mM CPZ	37/44	40/46	3/2	0.49	0.51	localized
(3) low salt, 4 mM TF	35/43	38/46	3/3	0.48	0.48	localized
(4) 100 mM KCl (high salt)	34/41	45/54	11/13	0.56	0.58	delocalized
(5) 100 mM KCl, 4 mM CPZ	37/46	51/61	14/15	0.54	0.51	delocalized

[a] Thylakoids were washed and resuspended in either the low-salt medium or the high-salt (100 mM KCl) medium as defined under Materials and Methods, or those media with the addition of chlorpromazine (CPZ) or trifluoperazine (TFP). Where present, pyridine was added at 4 mM, 3.5 min prior to starting the 5-Hz flash sequence. The energization lag was determined by the number of flashes before the first detectable rise in luminescence (number given before the slash) and by back-extrapolation of the steady-state rise in luminescence to the base line (number after slash). The column headed "lag difference" gives the criterion for deducing localized or delocalized coupling; i.e., when the presence of pyridine did not significantly increase the ATP formation onset lag parameters (as in samples 1–3), localized coupling is inferred. In samples 4 and 5, the pyridine effect on the lag parameters, being ≥11 flashes, is consistent with delocalized coupling. The standard deviations for the onset lags were not more than ±2 flashes, and for the ATP yield/flash not more than ±0.08 nmol of ATP (mg of Chl)$^{-1}$ flash^{-1}. Each result is the average of at least three assays.

or the number of flashes required to reach the energization threshold. Control experiments (not shown) using 5 mM CPZ in the phosphorylation assay confirmed the previous results (Good et al., 1966) that such high concentrations of CPZ inhibit ATP formation.

Effect of Phenothiazines on Postillumination ATP Formation. One interesting effect of CPZ or TF treatments in the storage stage was their selective effect on postillumination ATP formation (PIP). The great sensitivity of the luciferin–luciferase ATP monitoring system and the fact that the ATP measurement signal is detected in real time in the presence of the energized thylakoids allow direct detection of two types of PIP (Beard et al., 1988). The assay allows measurement of PIP, where ADP and P_i are present during the flash train, and PIP ATP yields assayed in this manner are referred to as PIP^+ to distinguish them from the "traditional" PIP^- ATP yields obtained when ADP and P_i are added after illumination (Hind & Jagendorf, 1986; Nelson et al., 1971, Avron, 1972). Horner and Moudrianakis (1983) were able to measure both types of PIP using rapid-quench techniques with the ^{32}P method. In agreement with Horner and Moudrianakis (1983), Beard et al. (1988) concluded that in 5-Hz flash trains, protons contributing to PIP^+ are accumulated in excess of what can be used in the 200-ms dark time between flashes and are released in the longer dark stage following the last flash. Under the conditions we use (10 °C), the $t_{1/2}$ for the PIP^+ ATP yield is near 1.2 s (Beard & Dilley, 1986), and that accounts for the incomplete dissipation of $\Delta\tilde{\mu}_{H^+}$ in the 200-ms time between flashes. For the PIP^- mode, no ATP formation occurs in the light, but proton uptake is active, resulting in a greater PIP^- ATP yield compared to the ATP yield under PIP^+ conditions (Figure 4).

As shown earlier (Beard & Dilley, 1986, 1988a), we can distinguish between localized and delocalized coupling mechanisms by two criteria: (1) the effect of a permeable amine (pyridine is commonly used) on the PIP^+ ATP yields and (2) the effect of the amine on the number of flashes required to initiate ATP formation. Regarding the PIP^+ parameter, and in agreement with the earlier results, low-salt-stored thylakoids show PIP^+ ATP formation, but there is no pyridine stimulation of the ATP yield (Figure 4, left side). Those results are consistent with localized energy coupling in which the energizing $\Delta\tilde{\mu}_{H^+}$ is built up in localized domains not accessible to the pyridine or having volumes too small for significant pyridine entry (Beard & Dilley, 1986; Beard et al., 1988). On the other hand, with high-salt-stored thylakoids, pyridine caused a significant increase in the PIP^+ ATP yield (Figure 4, left center), and that is consistent with delocalized coupling. Figure 4 also shows that neither CPZ nor TF had any effect on the PIP^+ yields with the low- or high-salt-stored thylakoids, so we can conclude that whatever residual drug may have remained in the thylakoids after dilution into the assay buffer, it was not sufficient to act as an inhibitor of ATP formation.

The second type of postillumination phosphorylation, PIP^-, is measured in the traditional manner (Hind & Jagendorf, 1963; Avron, 1972; Nelson et al., 1971; Ort et al., 1976; Vinkler et al., 1980). In that case, ADP and P_i were added *after* a flash train, and during the flash train, proton accumulation occurred under basal electron flow conditions. As reported earlier (Beard et al., 1988), *both low- and high-salt*-stored thylakoids show a large [≈10 nmol of ATP(mg of Chl)$^{-1}$] pyridine-dependent increase in the "traditional" PIP^- ATP yield (Figure 4). Our interpretation (Beard et al., 1988; Beard & Dilley, 1988a; Renganathan et al., 1991) is that

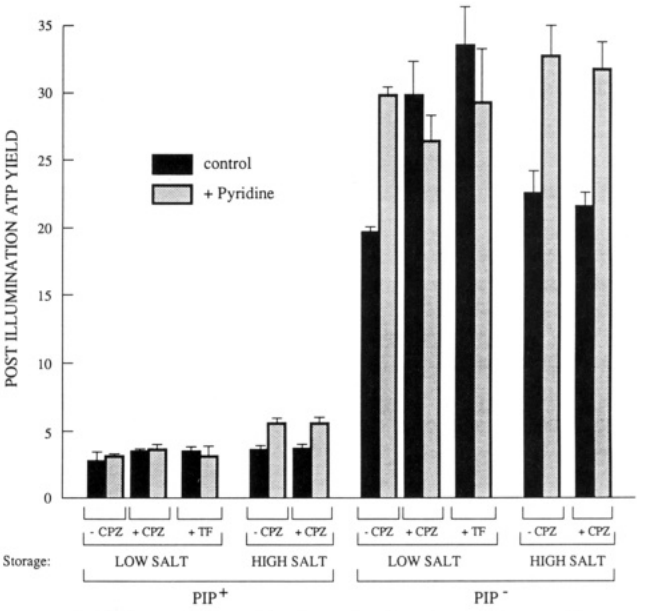

FIGURE 4: Effect of phenothiazines—in the storage stage only—on two types of postillumination phosphorylation in low- or high-salt-stored thylakoids. Thylakoids were washed and resuspended in either a low-salt or a high-salt medium as described under Materials and Methods. No UV treatment was given. Either chlorpromazine or trifluoperazine was added to the above resuspension mixtures as treatments during the thylakoid storage stage, prior to dilution into the assay media. The postillumination phosphorylation (see Materials and Methods for details) occurring after a flash train of 100 flashes when ADP and P_i were present during the flash excitation (referred to as PIP^+) was measured by the rise in luminescence occurring after the last flash [see Figure 1 of Beard and Dilley (1988b)]. Postillumination phosphorylation assayed in the traditional mode, referred to as PIP^-, was measured by the rise in luminescence when ADP and P_i were added after the last flash [see Figure 3 of Dilley (1991)]. Other components of the reaction mixture are described under Materials and Methods. In the PIP^- experiments, 100 flashes were delivered at 5 Hz. Each result is the average of at least three assays.

during energization by the basal electron flow, low-salt-stored thylakoids *do not constrain* the proton uptake to the localized domains but allow H^+ ion equilibration with the lumen. We suggest that in the absence of ATP formation-linked H^+ efflux, the accumulation of H^+ in the sequestered domains occurs in excess of the domain carboxyl group buffering capacity [discussed in Beard et al. (1988) and Beard and Dilley (1988a)], leading to a further lowering of the localized pH causing the postulated Ca^{2+}-controlled gate to open, allowing equilibration of the domain H^+ ion concentration with the lumen. Hence, the large pyridine-dependent PIP^- ATP yield increases with both low- and high-salt-stored thylakoids. With high-salt-stored thylakoids, chlorpromazine had no effect on the large pyridine-dependent PIP^- ATP yield increase (Figure 4), so that with or without the drug treatment a delocalized proton gradient was established and its collapse, with some protons coming from protonated lumenal pyridine, drove ATP formation. The lumenally-located pyridine results in an increased ATP yield of about 10 nmol of ATP (mg of Chl)$^{-1}$ over the control level of near 20 nmol of ATP (mg of Chl)$^{-1}$.

Surprisingly, however, the chlorpromazine-treated low-salt-stored thylakoids gave an increased PIP^- yield (Figure 4, right) in the *absence* of pyridine and *did not show* the pyridine-dependent increase in the PIP^- ATP yield characteristic of the control low-salt-stored case! Rather, pyridine caused a slight, perhaps not even signifmcant, decrease in the PIP^- ATP yield [compared with the pyridine-dependent increase of near 10 nmol of ATP (mg of Chl)$^{-1}$ in the low-salt control treatment]. In searching for an explanation for the surprising

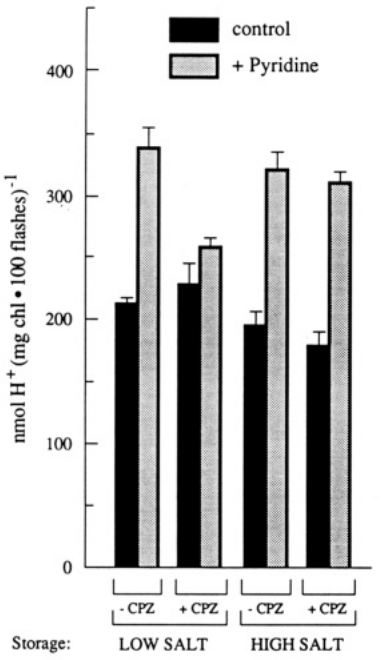

FIGURE 5: Effect of chlorpromazine—in the storage stage only—on lumenal H^+ uptake in low- and high-salt-stored thylakoids. See Materials and Methods for thylakoid preparation and H^+ uptake assay conditions. Basal conditions were used for H^+ uptake. Chlorpromazine at 5 μM final concentration was present in the thylakoid storage medium for at least 30 min prior to dilution by ≥150-fold into the H^+ uptake assay buffer, so that the CPZ concentration in the assay was ≤0.03 μM. When pyridine was used, the final concentration was 5 mM, and it was added 3.5 min before turning on the 5-Hz flashing light to initiate H^+ uptake.

effects of CPZ in the low-salt-stored case, one thought is that CPZ or TF could stimulate H^+ uptake into the lumen and therefore increase the PIP^- ATP yield. However, that explanation cannot hold because: (A) measurement of H^+ uptake under those conditions showed no significant increase in uptake in the low-salt case when CPZ was present in the storage stage (Figure 5); (B) there should also have been an additional CPZ plus pyridine-dependent stimulation of PIP^- ATP yield (since pyridine is known to cause greater lumenal H^+ uptake and PIP^- ATP yield, see above) and that was not observed; and (C) that effect was not observed with high-salt-stored thylakoids (Figure 4, right side). An alternative explanation for the above results and one completely consistent with the concept of Ca^{2+} gating of H^+ flux at the inner side of the CF_0 H^+ channel is that in the low-salt-stored case the phenothiazines bind to the Ca^{2+} gating site and block the release of Ca^{2+}. If Ca^{2+} is not displaced, we propose that it can block H^+ ions from flowing from the domains into the lumen.

Effect of Phenothiazines on Lumenal H^+ Uptake. A different assay for testing the postulate that CPZ or TF treatment of low-salt-stored thylakoids can block proton flux from the membrane domains to the lumen is to measure H^+ uptake into the lumen with and without Ca^{2+} antagonist pretreatment. For that experiment, proton uptake, driven by a 100-flash (5-Hz) excitation, was monitored with the pH electrode method (Dilley, 1972). The results of such an experiment are shown in Figure 5 for H^+ uptake under basal conditions at pH 7.5. It was necessary to use basal conditions for this experiment, because under coupled conditions the low-salt-stored thylakoids maintain localized H^+ uptake; i.e., there is not enough H^+ entry into the lumen to show significant pyridine stimulation, as was already shown by Beard et al. (1988) and Renganathan et al. (1991).

The well-known effect of amines stimulating lumenal H^+ uptake (Nelson et al., 1971; Avron, 1972) is shown in Figure 5 where, for the control case (no CPZ), the presence of pyridine caused nearly a 2-fold increase in basal electron transport-coupled H^+ uptake for *both low-* and *high-salt*-stored thylakoids. The striking result is that 5 μM CPZ given to high-salt-stored thylakoids (only in the storage stage) had no effect on the pyridine-dependent increase in H^+ uptake but with the low-salt-stored thylakoids the CPZ treatment *nearly completely blocked the pyridine-dependent extra H^+ uptake.* One must ask whether the CPZ inhibition of pyridine-dependent H^+ uptake in the low-salt condition could be explained by some type of increased H^+ efflux only in the CPZ-treated low-salt-stored thylakoids. A comparison of the kinetics of the H^+ decay rate constants (k_d), determined by the graphical method for analysis of a first-order process, showed that this was not the case. The CPZ-treated (storage stage) low-salt-stored thylakoids showed no increase in the H^+ decay rate compared to the control thylakoids (data not shown). In both the control and CPZ treatments, the k_d was 0.037 s^{-1}. This point was also supported by the observation that CPZ treatment did not decrease the efficiency of phosphorylation measured either by the length of the ATP formation onset lag or by the ATP yield per flash (Table V), as would be expected if the CPZ treatment had led to an increased H^+ efflux.

Discussion

These results give consistent support to the hypothesis that the CF_0 H^+ channel is a Ca^{2+}-regulated H^+ flux gate, functioning to gate H^+ fluxes between membrane-localized domains and the thylakoid lumen. The support for this concept rests on two separate lines of evidence: (1) structural data on the binding of a well-known Ca^{2+} binding site probe (chlorpromazine, CPZ) to the 8-kDa CF_0 subunit; (2) functional studies of H^+ fluxes and ATP formation showing that CPZ can block energy-linked H^+ fluxes from membrane-sequestered domains into the lumen. Additional supportive results to be presented separately (Wooten and Dilley, in preparation) show that CPZ can block most of the succinate-dependent acid–base-jump ATP formation in low-salt-stored thylakoids with no effect on the ATP yield with high-salt-stored membranes.

Structural Studies. The identity of the CPZ-labeled 8-kDa polypeptide as being CF_0 subunit III is supported by the following: (A) the high purification of the proteolipid achieved by the butanol/ether method with an SDS–PAGE step: (B) the coincidence of [^{14}C]DCCD and [^{3}H]CPZ in the same band of that preparation (Figure 2); and (C) the amino acid sequence data (Table I) obtained from the purified 8-kDa protein. DCCD labeling of the 8-kDa protein is not a unique labeling event in thylakoids, but it does give strong diagnostic evidence of the identity of the CF_0 protein, especially when the highly selective butanol/ether isolation procedure is used to extract and precipitate the protein prior to the SDS–PAGE step (Nelson et al., 1971). Moreover, it is known that labeling the 8-kDa CF_0 protein with DCCD changes the M_r value on SDS–PAGE gels just as we see in Figure 2 and as shown in earlier work (Tandy et al., 1982). Derivatization with DCCD also changes the column chromatographic elution behavior of the 8-kDa CF_0 protein (Doherty & Gray, 1980; Tandy et al., 1983; Hermolin & Fillingame, 1989). Chlorpromazine labeling of the 8-kDa protein does not change the SDS–PAGE M_r value (Figure 1B). Therefore, despite the lack of high counts of DCCD and CPZ in the main-stained 8-kDa band in the dual-labeled experiments of Figure 2, the labeling by both probes of the smaller band at the higher M_r can be taken as strong evidence for the 8-kDa CF_0 protein being a target

for CPZ photoaffinity labeling.

Further support for the notion that CPZ photoaffinity-labels the 8-kDa CF_0 subunit is the observation that [^{3}H]CPZ labels the 8-kDa band of the purified CF_0–CF_1 complex reconstituted into azolectin vesicles (Figure 3). That result indicates that the CPZ binding site is a property of CF_0 itself, and not dependent on a closely associated, but non-CF_0–CF_1 protein component. Dabbeni-Sala and Palatini (1990) and Dabbeni-Sala et al. (1990) have reported on CPZ photoaffinity labeling of the isolated, liposome-reconstituted F_0–F_1 from bovine heart. Their procedures resulted in labeling of the F_0 sector much more heavily than the F_1 subunits.

The identity of the 6-kDa polypeptide which copurified with the 8-kDa protein and which may be labeled with both CPZ and DCCD (Figures 1 and 2, Table I) is not known, but it clearly is not part of the 8-kDa protein as shown by the partial amino acid sequence (Table I). The results of a data base search with the GCG sequence analysis program shows significant similarity to a chloroplast gene-encoded hypothetical protein (see Results), but more work must be done to clarify this. From the present data, it cannot be ruled out that both the [^{3}H]CPZ and [^{14}C]DCCD counts in fractions 40 and 41 of Figure 2 (running as a shoulder ahead of the main 6-kDa band) may derive from a fragment of the 8-kDa protein. This is thought to be unlikely in view of the fact that the DCCD and CPZ labeling ratios are inverted when one compares fractions 29–31 (the 8-kDa material) to fractions 38–41 (near the 6-kDa band). Such an inversion in the labeling ratio is more consistent with the labeled protein in fractions 38–41 being a fast-running form of the 6-kDa protein reflecting a polypeptide different from the 8-kDa protein and with its own particular DCCD and CPZ labeling tendencies. It is possible that the dual-labeled band running ahead of the 6-kDa stained band is a fraction of the 6-kDa polypeptide, but with an altered mobility owing to the added ligands. The DCCD and CPZ counts in fractions 40 and 41 are not likely to be caused by a type of micelle containing the probes in free form, because the thylakoids were given the butanol extraction/ether precipitation treatment prior to SDS solubilization, and both probes in the free form are not likely to have accompanied the proteins through those steps. Future work must be done to clarify the identify and role of the 6-kDa polypeptide, but the speculative possibility is intriguing that it may be an additional Ca^{2+}-sensitive protein involved in functions related to proton processing.

A puzzling aspect of phenothiazine photoaffinity labeling of the 6- and 8-kDa components is the very low labeling level, roughly 1 CPZ per 10^4 8-kDa CF_0 molecules (Table II). Even so, the other membrane components were labeled much less. Clearly, the drug binding site is not comparable to, e.g., CPZ binding to (purified) calmodulin which occurs at nearly a 1:1 ratio of drug to protein (Prozialek et al., 1981). There is a report of an analogous low-[^{3}H]CPZ photoaffinity labeling of the ion channel of the acetylcholine receptor in the *Torpedo* electric organ (Oswald & Changeux, 1981). In that system and in the thylakoid system we are studying, the antagonist molecule binds with apparent high affinity, yet the photoaffinity labeling is not very efficient. In the thylakoid system, CPZ binding can be inferred as high affinity (we have not quantitatively measured an affinity constant) owing to the nearly complete blocking of domain H^+ entry into the lumen (Figure 5) and the complete blocking of the pyridine-dependent PIP^- ATP yield (Figure 4). It may be that the UV-formed radical species of the CPZ occupies its binding site such that the radical can degrade with higher efficiency then reacting with proteins in the binding site, as suggested by Oswald and Changeux (1981). With calmodulin, the complex may be situated more favorably for cross-linking. In any event, it is clear that thylakoid CPZ binding is not to a calmodulin-type protein. Although plants contain calmodulin (Roberts et al., 1986), chloroplasts contain very little, if any (Simon et al., 1984), even though there are calmodulin-like Ca^{2+}-dependent enzymes in chloroplasts (Kreimer et al., 1987).

A puzzling feature of these results is that the CPZ labeling pattern in the low-salt-stored, compared to the high-salt-stored, thylakoids was more variable and the differences were less dramatic than the CPZ effects observed in the functional assay pattern. In the ATP formation (Figure 4, Table V) or H^+ uptake (Figure 5) assays, the two thylakoid preparations give very reproducible and decidedly different responses, and those responses form the basis on which we have postulated the Ca^{2+}-regulated coupling between localized or delocalized modes. CPZ labeling of the 6- and 8-kDa polypeptides in the low- and high-salt-stored thylakoids differed by only a factor of 2–3 (Tables II and III), or less in some experiments, yet the functional responses to CPZ were essentially all or none. Perhaps the different responses are related to the fact that the UV labeling experiments involved 20 min or more for UV exposure while the functional assays were completed in 5 min or less.

Another aspect which may influence the CPZ labeling results in a way not yet understood is the observation that H^+ uptake occurring during UV illumination greatly decreased the CPZ labeling in both types of thylakoids (Table IV). One possibility is that H^+ uptake during UV treatment, while rather low [<100 nmol of H^+ (mg of Chl)$^{-1}$], could protonate groups (i.e., COO^- groups?) normally involved in binding Ca^{2+} ions, resulting in displacement of the Ca^{2+} and a decreased affinity for CPZ binding. Further work is needed to clarify the relationships between Ca^{2+} effects on H^+ fluxes associated with energy coupling and the suggested involvement of the CF_0 structure. In any event, the speculations which follow serve to focus attention on some possible testable models.

Although CPZ and other phenothiazines are valuable research tools owing to their high affinity for the Ca^{2+}-occupied form of tight Ca^{2+} binding proteins (Prozialek et al., 1981), there are other possible interactions of these amines with biological materials; i.e., they are uncouplers (Good et al., 1966), and they can act as local anesthetics (Oswald & Changeux, 1981), neither of which action is necessarily dependent on Ca^{2+} or Ca^{2+} protein adducts as far as we are aware. Therefore, care must be taken to avoid an erroneous assignment of these probes as probes for putative Ca^{2+} binding sites. However, in support of the notion that a Ca^{2+} binding protein site is involved here, CPZ appears to be interacting with the 8-kDa CF_0 protein with a Ca^{2+} dependence. Thus, in the experiment described in Table III, adding $CaCl_2$ to high-salt-stored thylakoids increased CPZ photoaffinity labeling. That correlates well with our previous functional studies (Chiang & Dilley, 1987) in which adding similar levels of $CaCl_2$ to "delocalized responding" high-salt-stored thylakoids converted them to "localized responding thylakoids." Additional support for CPZ labeling of the 8-kDa CF_0 subunit being enhanced by Ca^{2+} is seen in Table IV, where adding an external Ca^{2+} trap (EGTA) and a divalent cation ionophore (A23187, to enhance Ca^{2+} transfer from the lumen to the outside) caused a significant decrease in the [^{3}H]CPZ labeling of the 8-kDa protein.

Table IV also indicates that energizing the thylakoids decreased the 8-kDa and 6-kDa CPZ labeling. The mechanism

of this effect is not understood, but it seems related to H^+ accumulation (energization) and, because the effect was also observed in the presence of the electron-transport inhibitor DCMU, the effect is not a function of electrochemical reduction of CPZ by the electron transfer supported by the UV-C light source used. The energized state could cause Ca^{2+} to be displaced from some of the CF_0 binding sites by competititon of the accumulated H^+ ions for the putative carboxyl binding sites (Dilley, 1991), or energization may alter the conformational state of the CF_0 complex in a way that decreases the likelihood of CPZ forming a proper complex with the 8-kDa subunit. These speculations await future work to test.

Functional Studies. A second and very important line of evidence supporting the Ca^{2+} gating hypothesis is the finding that CPZ or TF added to the low-salt-stored thylakoids caused an apparent blocking of H^+ ion entry into the lumen (Figure 5). Lumenal H^+ accumulation was detected by pyridine-dependent extra H^+ uptake, an accepted criterion for lumenal H^+ uptake (Nelson et al., 1971; Avron, 1972). With high-salt-stored thylakoids, there was no influence of CPZ on pyridine-dependent lumenal H^+ uptake (Figure 5), and that is consistent with a prediction based on phosphorylation studies that high-salt-stored membranes have the putative Ca^{2+}-regulated gate in the *open* configuration, allowing H^+ entry to the lumen. Of importance was the finding that CPZ present only in the thylakoid storage stage did not cause any increase in the H^+ efflux rate constant or any uncoupling, the latter point based on the criteria of ATP yield per flash and the length of the ATP onset lag (Table V).

If, with low-salt-stored thylakoids, CPZ blocks H^+ entry into the lumen under the conditions used (5-Hz single-turnover flashes), it would be predicted that with the traditional type of postillumination ATP formation (PIP^-), the *pyridine*-dependent part of the PIP^- ATP yield would also be inhibited by CPZ. That was observed to occur (Figure 4), in the low-salt-stored thylakoids, whereas no inhibition was seen in high-salt-stored membranes, exactly corresponding to the CPZ effects on H^+ uptake into the lumen with thylakoids from the two storage conditions.

However, we must comment on the observation that while blocking the pyridine stimulation of H^+ uptake in low-salt-stored membranes (Figure 5) and thus blocking the pyridine-dependent PIP^- ATP yield, the phenothiazine treatment stimulated the (minus pyridine) control PIP^- yields (Figure 4). Because the total H^+ uptake was not diminished by CPZ (Figure 5), but lumenal uptake apparently was decreased, it must be that in that case more H^+ ions accumulated in the domains. The proposed increase in domain H^+ accumulation caused by CPZ in the absence of pyridine neatly explains the otherwise inexplicable increased PIP^- ATP yield (Figure 4) observed in the CPZ-treated low-salt-stored thylakoids. The interesting implication here is that domain-accumulated protons may be, in some way, more effective than the equivalent amount of lumenal protons in driving PIP^- ATP yields.

Speculations on a Model. The models shown in Figure 1 of Dilley and Chiang (1989) or Figure 1 of Dilley (1991) express the concept of a Ca^{2+}-gated H^+ flux pathway connecting localized H^+ domains with the CF_0–CF_1 complex and with the lumen. The results presented here suggest that the 8-kDa CF_0 subunit is a part of the putative gating structure. As discussed in more detail elsewhere (Chiang & Dilley, 1987; Dilley & Chiang, 1989; Dilley, 1991), the Ca^{2+} effects on energy coupling modes are readily reversible. The Ca^{2+} can be displaced from the putative binding site (assayed indirectly by observing either localized or delocalized energy coupling or lumenal H^+ accumulation) by high K^+ or Na^+ levels (Beard & Dilley, 1988a), by Ca^{2+} chelators which can permeate across the membrane (Chiang & Dilley, 1987), or by excessive H^+ accumulation in the membrane domains under basal conditions as in Figure 5 of this work [see also Beard et al. (1988), Table III, and Renganathan et al. (1991), Figure 3]. Under basal conditions, we suggest that the excessive accumulation of domain H^+ ions permits the local domain pH to become acidic enough to protonate the putative gate carboxyl groups, allowing Ca^{2+} to diffuse away and the gate to open. For example, with pH 8 conditions outside, and given that the thermodynamically calculated threshold pH in the domains needed to initiate ATP formation is near pH 5.7 (Beard & Dilley, 1988a), if we suppose that the "Ca^{2+} gate" carboxyl groups have pK_a values near or below pH 5 [Horner and Moudrianakis (1986) proposed, in a general way, a similar scheme], then it can be pictured how, under basal conditions, the *excess* domain H^+ uptake could cause gate opening. Given the steep dependence of the rate of ATP formation with increasing ΔpH (Rottenberg et al., 1972; Davenport & McCarty, 1986; Gräber, 1982), it is possible that (particularly in 5-Hz flashes) the sequestered domain pH would not necessarily drop much below the threshold pH for energizing ATP formation. If the pK_a of the proposed Ca^{2+} binding carboxyl groups at the gate site was, e.g., near pH 5 or lower, then it can be understood that localized H^+ gradients may be maintained during ongoing ATP formation. Recent results (Renganathan et al., 1991) with steady-illumination ATP formation assays support this suggestion, as do other reports of localized coupling in steady illumination of duration near or longer than 1 min (Pick et al., 1987; Sigalat et al., 1985). However, in the absence of ADP and P_i, or if the rate of the H^+ release steps exceeds the capacity of CF_0–CF_1 to conduct H^+ outward in the coupling reactions, then we can speculate that the pH in the domains could drop to the range of pH 5 or lower and cause protonation of the proposed Ca^{2+} binding carboxyl groups, with opening of the H^+ flux gate to the lumen. As shown previously, both localized and delocalized energy coupling (as we have defined them by our assays) occur with equal efficiency once the threshold energization is reached (Beard & Dilley, 1986, 1988a). The ideas discussed here are speculative possibilities, and we recognize them as such, and hope that they will stimulate new experiments. Several aspects are amenable to experimental testing. Speculations on the possible biological advantages accruing to thylakoids in having both localized and delocalized energy coupling modes (with a regulated switch) have been given elsewhere (Dilley et al., 1987; Dilley, 1991).

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