Journal of Bioenergetics and Biomembranes, Vol. 25, No. 5, 1993

Calcium Gating of H^+ Fluxes in Chloroplasts Affects Acid–Base-Driven ATP Formation

Dennis C. Wooten[1] and Richard A. Dilley[1,2]

Received February 1, 1993

In previous work, calcium ions, bound at the lumenal side of the CF_0H^+ channel, were suggested to keep a H^+ flux gating site closed, favoring sequestered domain H^+ ions flowing directly into the CF_0–CF_1 and driving ATP formation by a localized $\Delta\tilde{\mu}_{H^+}$ gradient. Treatments expected to displace Ca^{++} from binding sites had the effect of allowing H^+ ions in the sequestered domains to equilibrate with the lumen, and energy coupling showed delocalized characteristics. The existence of such a gating function implies that a closed-gate configuration would block lumenal H^+ ions from entering the CF_0–CF_1 complex. In this work that prediction was tested using as an assay the dark, acid–base jump ATP formation phenomenon driven by H^+ ions derived from succinic acid loaded into the lumen.

Chlorpromazine, a photoaffinity probe for many proteins having high-affinity Ca^{++}-binding sites, covalently binds to the 8-kDa CF_0 subunit in the largest amounts when there is sufficient Ca^{++} to favor the localized energy coupling mode, i.e., the "gate closed" configuration. Photoaffinity-bound chlorpromazine blocked 50% or more of the succinate-dependent acid–base jump ATP formation, provided that the ionic conditions during the UV photoaffinity treatment were those which favor a localized energy coupling pattern and a higher level of chlorpromazine labeling of the 8-kDa CF_0 subunit. Thylakoids held under conditions favoring a delocalized energy coupling mode and less chlorpromazine labeling of the CF_0 subunit did not show any inhibition of acid–base jump ATP formation.

Chlorpromazine and calmidazolium, another Ca^{++} -binding site probe, were also shown to block redox-derived H^+ initially released into sequestered domains from entering the lumen, at low levels of domain H^+ accumulation, but not at higher H^+ uptake levels; ie., the closed gate state can be overcome by sufficiently acidic conditions. That is consistent with the observation that the inhibition of lumenal succinate-dependent ATP formation by photoaffinity-attached chlorpromazine can be reversed by lowering the pH of the acid stage from 5.5 to 4.5.

The evidence is consistent with the concept that Ca^{++} bound at the lumenal side of the CF_0 H^+ channel can block H^+ flux from either direction, consistent with the existence of a molecular structure in the CF_0 complex having the properties of a gate for H^+ flux across the inner boundary of the CF_0. Such a gate could control the expression of localized or delocalized $\Delta\tilde{\mu}_{H^+}$ energy coupling gradients.

KEY WORDS: Energy coupling; ATP formation; H^+ gradients; Ca^{++} binding in thylakoids.

INTRODUCTION

Energy-transducing membranes in chloroplasts, mitochondria, and most bacteria utilize H^+ gradients to drive ATP formation (Ferguson, 1985; Boyer *et al.*, 1977). Several fundamental aspects of how H^+ ions interact with membranes in the establishment and utilization of the $\Delta\tilde{\mu}_{H^+}$ in energy coupling are not understood, and one issue involves the possibility of membrane localization of the $\Delta\tilde{\mu}_{H^+}$ gradient. Bulk phase-to-bulk phase $\Delta\tilde{\mu}_{H^+}$ coupling to ATP form-

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

0145-479X/93/1000-0557$07.00/0

ation is generally accepted as a valid mechanism (Ferguson, 1985), but for all three coupling membrane systems there have been reports of ATP formation being driven, in some instances, by a type of localized $\Delta\tilde{\mu}_{H^+}$; in mitochondria, see Ferguson (1985) for a broad review, Rottenberg (1985), Mandolino *et al.* (1983), and Luvisetto *et al.* (1990); for chloroplasts, see Ort *et al.* (1976), Horner and Moudrianakis (1983), Beard and Dilley (1986, 1988), Pick *et al.* (1987), Sigalat *et al.* (1985); for bacteria, see Melandri *et al.* (1980), van Walraven *et al.* (1984), and Guffanti and Krulwich (1988). However, the diverse reports supporting localized $\Delta\tilde{\mu}_{H^+}$ energy coupling leave unanswered two important questions, one concerning the nature of the putative localized domains and how H^+ ions might move in them, and the other about the regulatory factors that must be present to allow the CF_0 (or the F_0?) H^+ chanel to be linked to and switched between either the localized or the delocalized H^+ source.

Our work with thylakoids has shed some light on the latter question inasmuch as Chiang and Dilley (1987) have shown that Ca^{++} ions appear to act as a gating factor in (reversibly) switching between experimentally detectable localized or delocalized $\Delta\tilde{\mu}_{H^+}$ energy-coupling gradients. For example, storing thylakoids in buffer containing 100 mM KCl or in a low-salt medium with membrane-permeable Ca^{++} chelators correlates with observing delocalized energy coupling. Storing thylakoids in a low-salt medium, or in the 100 mM KCl medium with 1 mM $CaCl_2$ or 0.1 mM $LaCl_3$, correlates with observing a localized energy coupling pattern. Other data suggested that the proposed gating Ca^{++} acts at or near the 8-kDa CF_0 subunit of the H^+ channel (Chiang and Dilley, 1988; Chiang *et al.*, 1992). Evidence for this notion came from experiments with chlorpromazine, a photoaffinity probe for tight Ca^{++}-binding sites on a variety of proteins (Prozialeck *et al.*, 1981). The probe was shown to photoaffinity label the 8-kDa CF_0 subunit in a Ca^{++}-dependent way, and in thylakoids committed to the localized coupling mode, chlorpromazine blocked H^+ ions, accumulated in membrane domains, from entering the lumen and blocked the component of postillumination ATP formation dependent on lumenally accumulated buffers (Chiang and Dilley, 1988; Dilley and Chiang, 1989).

The hypothesis we currently are considering posits that the lumenal side of the CF_0 H^+ channel has a high-affinity Ca^{++}-binding site that, in an as-yet-unknown way, involves the 8-kDa protein(s) such that when Ca^{++} occupies the site—as in thylakoids stored in a low-salt medium—H^+ ions released by redox reactions into sequestered domains accumulate in the domains to levels near 150 nmol H^+/mg Chl and reach a pH acidic enough (pH < 6.0) to drive ATP formation with the lumen pH remaining above pH 7.0 (Renganathan *et al.*, 1993). However, in such thylakoids, domain H^+ accumulation can be induced to equilibrate with the lumen, for instance, under basal electron transport conditions in low-salt stored thylakoids, when ADP is not present to permit coupling of H^+ efflux to ATP formation (Beard *et al.*, 1988; Renganathan *et al.*, 1991). On the other hand, conditions conducive with displacing Ca^{++} from binding sites, such as storing thylakoids in high KCl, or in the low-salt medium with the addition of membrane-permeable Ca^{++}chelators (Chiang and Dilley, 1987) resulted in energization always giving lumenal H^+ accumulation in either coupled or basal conditions (Renganathan *et al.*, 1993). The apparent gating mechanism is not well understood, though, because the KCl treatment in the dark does not, by itself, induce equilibration of domain protons with the lumen (Allnutt *et al.*, 1989), implying that the high KCl simply potentiates the system so that some response to light, electron transport, or low levels of H^+ ions released in the redox reactions could be required to complete the gate opening. One way to test whether light or electron transfer are required factors, *per se*, in this system is to do an assay under dark conditions that can test for the open or closed gate configuration.

The dark acid–base jump ATP formation protocol, using lumenally loaded succinic acid as a source of protons (Jagendorf and Uribe, 1966), provides such an assay, and it will be used herein in conjunction with photoaffinity-attached chlorpromazine.

METHODS

Materials

Chlorpromazine-HCl, P_1, P_5-[diadenosine-5′]-pentaphosphate, DCMU,[3] succinic acid, glutamic acid, and defatted bovine serum albumin were obtained from Sigma Chemical Company.

[3] Abbreviations used: Chl, chlorophyll; CPZ, chlorpromazine; DCMU, *N*-(3,4-dichlorophenyl)-*N*, *N*-dimethylurea; HEM, hydroxylethylmorpholine; MV, methyl viologen.

Chloroplast Isolation

Thylakoids were prepared and supended in either a low-salt or high-salt medium as previously described (Beard and Dilley, 1986), from 14- to 21-day-old pea (*Pisum sativa,* var. Little Marvel) plants, grown with supplemental light.

Chlorpromazine Photoaffinity Binding to Thylakoids

Prior to UV exposure, 10 μM chlorpromazine in MeOH was added to a portion of the stock thylakoid suspension at 2.5 mg Chl/ml and incubated for 30 min at ice bath temperature. The controls received MeOH only. The thylakoids were then diluted to 33 μg Chl/ml with either the low- or high-salt medium (but without the BSA), and the treated sample was brought to 10 μM CPZ. The diluted thylakoid suspensions were placed in open specimen dishes in a 4°C ice bath and either kept in darkness or exposed to 10 min of UV light from a model UVL-21 Blak-Ray lamp (UVP Inc., San Gabriel, California) placed 4 cm above the stirred samples. After the treatment, the thylakoids were centrifuged at 3000 × *g* for 4 min and resuspended in fresh low- or high-salt medium (without chlorpromazine) to a Chl concentration of 3–4 mg Chl/ml.

Acid–Base Transition ATP Formation

Chloroplast thylakoids (30μg Chl) were added to 0.9 ml of acid stage medium containing 20 mM succinic acid, 5 mM glutamic acid, 40 μM DCMU, 0.9 μM valinomycin, 22 μM P_1, P_5-[diadenosine-5′]pentaphosphate, 10 mM sorbitol, 5 mM MgC_2, and appropriate amounts of 20 mM NaOH for pH adjustment. The acid incubation time was 30 sec for pH 4.0 and 2 min for pH 5.5. After the acid stage, 0.9 ml of the base stage medium was added containing 110 mM Tricine, 2 mM K_2HPO_4, 22μM P_1,P_5-[diadenosine-5′]pentaphosphate, 0.2 mM ADP, and sufficient KOH for pH adjustment to a final pH of 7.5 or 8.5, depending upon the acid stage pH. Acid–base transitions were done at 10°C. The thylakoids were then rapidly pelleted in a microcentrifuge and the supernatant was collected for later assay of the ATP yield.

Determination of ATP Yields

ATP yields were determined by the luciferin–luciferase technique using the LKB-ATP monitoring kit with the reagent made up according to Dilley and Schreiber (1984). The luminescence-detection was carried out at 10°C in a thermostated cuvette, which was connected by a light guide (shielded by a Corning 5-96 filter) to a Hamamatsu R562 photomultiplier tube, powered by a Hewlett-Packard 6515A power supply. The signal was amplified by a home-built current converter (100 nA/v), and fed into a strip chart recorder. Ten μl of the LKB luciferin–luciferase solution was added to the stirred cuvette chamber containing 0.9 ml of a buffer consisting of 10 mM sorbitol, 50 mM Tricine-KOH, pH 8.0, 3 mM $MgCl_2$, and 4 mM KH_2PO_4. Calibration was done by adding 5 μl of LKB ATP standard before adding an aliquot of the acid–base transition supernatant. A volume of 80 μl of the acid–base transition supernatant was added to determine its ATP content. At least three separate assays of each supernatant solution were done.

H^+ Uptake Assays

Light-driven H^+ uptake in thylakoids was measured as described in Renganathan *et al.* (1991) using the $H_2O \rightarrow$ MV electron transfer system. The assay medium consisted of 1 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 20 mM KCl, 100 mM sorbitol, 0.1 mM methyl viologen, 0.2 μM nonactin, and 20 μg/ml chlorophyll, and the temperture was kept at 10°C. The pH was measured with an Orion combination pH electrode connected to a Corning Model 12 pH meter and the signal recorded on a strip chart recorder. Calibration of the pH changes was done by addition of standard HCl.

Illumination of the water-jacketed reaction vessel was provided by a 500-watt projector lamp focused through a $CuSO_4$-containing round bottom flask and passed through a Corning CS-2-64 (2030) red filter. Light intensity was measured with a Licor Model LI-189 light meter which measures photosynthetically active radiation (400–700 nm).

RESULTS

Chlorpromazine is known to inhibit ATP formation (Good *et al.*, 1966; Chiang, Ph.D. Thesis, 1989), but the noncovalently linked inhibitor at 10 μM could be readily washed out by centrifugation and resuspension in fresh medium prior to the acid–base jump (Table I). This allowed photoaffinity attachment of chlorpromazine to thylakoids in a pretreatment followed by a washing step prior to carrying out the acid–base jump protocol. Obviously, any chlorpromazine inhibition observed after the photoaffinity treatment would not be owing to carryover of free

Table I. Lack of Effect of UV Exposure or Chlorpromazine as Pretreatments on Acid–Base Jump ATP Formation[a]

	Pretreatments		Acid–base jump conditions		
	UV	Chlorpromazine	Acid used	ΔpH	ATP yield (nmol (mg Chl)$^{-1}$)
1.	—	—	Succinate	5.5 → 8.5	75 ± 6
2.	+	—	Succinate	5.5 → 8.5	77 ± 4
3.	—	—	Succinate	4.0 → 8.0	100 ± 2
4.	—	+	Succinate	4.0 → 8.0	102 ± 2
5.	—	—	HCl	4.0 → 8.0	19 ± 1

[a] Low-salt stored thylakoids were given UV treatment of 10 μM chlorpromazine, as described in Methods, as pretreatments, followed by centrifugation and resuspension in fresh medium for the acid–base jump assay (cf. Methods). The acid–base jump ATP formation assay was done as described in Methods. The acid stage had 20 mM succinate (lines 1–4) or only HCl (line 5). A minimum of three separate samples were used for each condition listed.

chlorpromazine from the 10μM pretreatment, but caused by the covalently linked compound, as shown in Table II and discussed below.

Table I shows that the UV treatment used to photoactivate the chlorpromazine was not, in itself, inhibitory. The table also indicates the expected increase in acid–base jump ATP formation owing to an acid stage of 4.0 compared to 5.5 and to succinic acid used in the acid stage compared to HCl (Jagendorf and Uribe, 1966). The latter point is evidence for the accumulation of succinic acid in the lumen space during the acid incubation, and the increased ATP yield can be attributed to H^+ ions originating from the succinic acid dissociating in the lumen during the alkaline stage and effluxing through the CF_0–CF_1 to the outside. Effects on the succinic acid-dependent portion of the ATP yield can, therefore, be a handle on the putative H^+ flux gate.

It is important to point out that we *do not assume* that the acid–base ATP yield from low-salt stored thylakoids originates from the *sequestered domains*. Rather, we assume that the pH 5.5 or below conditions in the acid stage cause the putative H^+ flux gate on the CF_0 to open, allowing lumenal H^+ ions, particularly those originating from the lumenally located succinic acid (Uribe and Jagendorf, 1967), to efflux through the CF_0–CF_1 and drive ATP formation. That is, both low- and high-salt thylakoids are forced into a delocalized energy coupling mode by the ≤ pH 5.5 conditions, hence the large succinate-dependent acid–base ATP yield oserved in Table I. As Jagendorf and Uribe (1966) first showed, succinic acid gives 4–5 times more ATP yield than HCl, and it was clearly shown that succinic acid goes to the lumen in the acid stage (Uribe and Jagendorf, 1967).

A similar point was made concerning postillumination phosphorylation (PIP) by Beard *et al* (1988) and Renganathan *et al.* (1991), namely that low-salt stored (or high-salt) thylakoids when energized by electron transport in the *absence* of ADP cause a large lumenal H^+ accumulation which can drive a large PIP ATP yield and it is greatly stimulated by a buffer such as (lumenally located) pyridine, analogous to the succinic acid in the acid–base experiment.

These comments should help set the stage for the next, key, experiment wherein chlorpromazine influences the acid–base ATP yield in a fashion understandable in terms of effects on the putative Ca^{++}-CF_0 H^+ flux gate.

Low-salt stored thylakoids exposd to chlorpromazine and UV light during pretreatment showed about 60% inhibition of acid–base jump (pH 5.5 → 8.5) ATP formation, whereas the treatment had no effect on high-salt stored membranes (Table II). Adding 1.5 mM $CaCl_2$ to the 100 mM KCl (high-salt) storage and chlorpromazine treatment media resulted in 39% inhibition of the ATP formation driven by a lumenally derived H^+ gradient. Previous results showed that thylakoids stored in low-salt conditions or in high-salt storage plus 1.0 mM $CaCl_2$ had a localized, $\Delta\tilde{\mu}_{H^+}$ gradient-driven ATP formation and a relatively high level of [^{3}H]chlorpromazine labeling of the 8-kDa CF_0 subunit, whereas high-salt stored thylakoids (or low-salt storage plus a membrane-permeable Ca^{++} chelator) had a delocalized $\Delta\tilde{\mu}_{H^+}$ energy coupling pattern and about one-half the level of chlorpromazine labeling of the 8-kDa protein (Chiang and Dilley, 1987; Chiang *et al.*, 1992). The effects of photoaffinity-bound chlorpromazine on acid–base jump ATP formation shown in Table II are consistent with the earlier results in that conditions favoring Ca^{++} occupancy of the hypo-

Table II. Effect of Photoaffinity-Activated Chlorpromazine Treatment on Acid–Base Jump ATP Formation[a]

	Conditions for thylakoid storage and UV treatment	Chlorpromazine	Acid–base jump ATP yield (nmol (mg Chl)$^{-1}$)	% Inhibition
1.	Low salt	—	77 ± 8	—
		+	30 ± 4	61%
2.	High salt	—	76 ± 7	—
		+	76 ± 4	0%
3.	High salt + 1.5 mM $CaCl_2$	—	51 ± 5	—
		+	31 ± 5	39%

[a] The procedures were as in Table I, except that UV treatments were given as described in Methods. Chlorpromazine was 10 μM where indicated and it was present only in the pretreatment stage. After the UV and chlorpromazine treatments, the thylakoids were centrifuged and resuspended in fresh medium (with no chlorpromazine) and assayed for acid–base jump (pH 5.5 → 8.5) ATP formation as described in Methods. A minimum of three separate samples were used for each condition.

thesized gating site on the CF_0 also favor chlorpromazine binding, and more drug binding corresponds to greater inhibition of lumenal H^+-driven acid–base jump ATP formation. The results are consistent with a gating effect on H^+ flux into the CF_0 channel. As a control measure, we tested representative thylakoid preparations used for the photoaffinity treatments for their localized or delocalized energy coupling responses.

Representative thylakoid samples used for these experiments were tested for the effect of the succinic acid loading step on their energy coupling responses in the single-turnover flash-driven ATP formation onset assay routinely used to assess localized or delocalized energy coupling behavior (Beard and Dilley, 1988). The assay measures the effect of the permeable buffer pyridine on the number of flashes required to initiate ATP formation. The effect of 35 mM succinate and pH 5.5 incubation was to increase the ATP formation onset lag by about 7–8 flashes, but that occurred equally for the samples with or without pyridine. Moreover, in the low-salt stored thylakoids 5 mM pyridine had only a slight effect on the number of flashes required to reach the energetic threshold (2–5 flashes) whereas in the high-salt stored sample 5 mM pyridine gave a 10–12 flash increase (data not shown). By that criterion, the succinic acid-loaded thylakoids maintained localized or delocalized energy coupling responses for low- or high-salt stored samples, respectively.

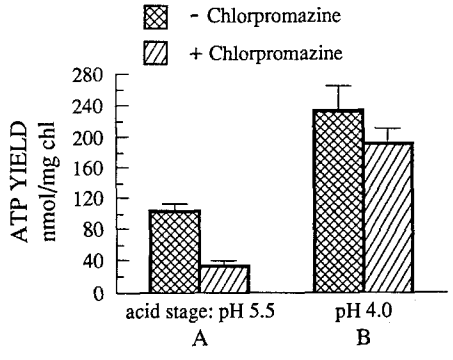

Fig. 1. The pH dependence of the effects of covalently bound chlorpromazine on acid–base jump ATP yield in low-salt stored thylakoids. Procedures were as in Table II. Acid–base transitions were from either (A) pH 5.5 to pH 8.5 or (B) from pH 4.0 to pH 7.5. The thylakoids were stored in low-salt storage medium containing 1.5 mM $CaCl_2$, and were exposed to ultraviolet light in low-salt exposure medium in the presence or absence of 30 μM chlorpromazine and 1.5 mM $CaCl_2$. Each column and bar is the mean and standard deviation of three experiments.

The chlorpromazine inhibition of acid–base jump ATP formation in the low-salt stored thylakoids was nearly completely reversed when the pH of the acid stage was dropped to 4.0 compared to pH 5.5 (Fig. 1). As expected, the lower pH gave a much greater yield of ATP owing to there being more protonated succinic acid loaded into the lumen at the lower pH (Jagendorf and Uribe, 1966). The failure of chlorpromazine binding to block part of the acid–base jump ATP formation when the acid stage was pH 4 was not caused by the 30 sec at pH 4 releasing the bound chlorpromazine, as shown by control experiments with the tritiated compound.

Thylakoids with covalently bound [^{3}H]chlorpromazine were prepared as described by Chiang *et al.* (1992), and aliquots were taken to pH 4.0 for a 1 min incubation in the medium used for the experiments described in Fig. 1, then returned to pH 7.5 as in an acid–base jump experiment. The control thylakoids were kept at pH 7.5. The labeling of the thylakoids was not changed by the pH 4 treatment. The control gave 164 ± 2 CPM/μg thylakoid protein and the pH 4 treated sample gave 163 ± 2 (in both cases the samples counted gave about 4000 CPM).

The chlorpromazine inhibition of the succinate-dependent acid–base jump ATP formation in low-salt stored thylakoids observed at pH 5.5 for the acid stage could also be reversed by simply washing the photoaffinity-treated thylakoids in the high-salt medium (Fig. 2).

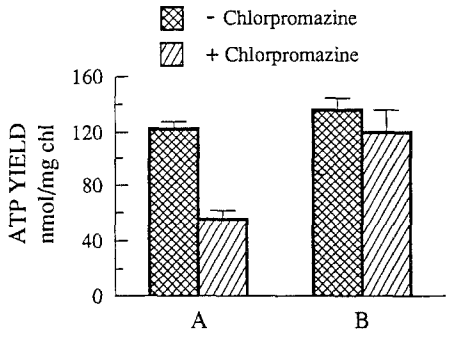

Fig. 2. The reversibility of the effects of covalently bound chlorpromazine, in low-salt + calcium-treated thylakoids, by washing and resuspension in high-salt medium without $CaCl_2$. Procedures were described under Methods. Acid–base transitions were from pH 5.5 to pH 8.5. Low-salt thylakoids were exposed to ultraviolet light in the presence of 10 μM chlorpromazine and 1.5 mM $CaCl_2$. After UV exposure, the thylakoids were washed and resuspended in either (A) low-salt + 1.5 mM $CaCl_2$ storage medium or (B) high-salt storage medium without Ca^{2+} and then given the acid–base jump ATP formation procedure. Each column and bar is the mean and standard deviation of three experiments.

Evidence for Gating in H^+ Uptake Assay

The foregoing data are consistent with chlorpromazine blocking a H^+ flux channel leading from the lumen though the CF_0–CF_1 complex. Another way to test for the existence of a gating site would be to observe gating action on H^+ ions *entering* the lumen, either from electron transport-linked or ATPase-linked H^+ pump activity. Such a gating action was demonstrated in preliminary findings by Chiang and Dilley (1988) using redox-linked H^+ pump activity, and here we develop the point further.

The assay used for this experiment was the effect of lumenally located amines on the extent of H^+ uptake. It is accepted that the stimulation of H^+ uptake by amines such as pyridine, pK 5.4, or hydroxyethylmorpholine, pK 6.2 (at less than uncoupling concentrations), is caused by part of the lumenally accumulated H^+ ions being buffered by the amine, allowing for a greater H^+ uptake to occur before the pH drops low enough to bring the pump and the leak into balance (Avron, 1971; Nelson *et al.*, 1971; Pick and Avron, 1976).

It should be noted that low-salt stored thylakoids under basal (–ADP) conditions were used for these experiments in order to saturate the H^+ buffering capacity of the domains, estimated to be near 120–150 nmol H^+ (mg Chl)$^{-1}$ (Beard *et al.*, 1988; Renganathan *et al.*, 1991), and induce H^+ deposition into the lumen. Moreover, by using low-salt stored membrane we expect that in the dark, unenergized state, Ca^{++} should occupy the CF_0 gating site and that, of course, is the postulated target of the chlorpromazine or calmidazolium drugs. Our working hypothesis is that the saturation of the domain buffering capacity leads to much more acidic conditions in the domains (compared to coupled conditions, where ATP formation "bleeds off" domain protons) which causes the putative H^+ flux gate to open.

For this experiment we used calmidazolium (without UV exposure) and UV photoaffinity-attached chlorpromazine. Calmidazolium, another probe for Ca^{++}-binding proteins, has a different structure than the phenothiazine class to which chlorpromazine belongs, providng a broader perspective on the putative thylakoid Ca^{++}-binding proteins. Figure 3 shows that calmidazolium treatment of low-salt stored membranes (60 μM, present in the storage stage only) nearly completely blocked the HEM amine-dependent component of H^+ uptake at low $\leq$ 14 μEin m^{-2} s^{-1}) light intensities, giving up to 120 nmol H^+ (mg Chl)$^{-1}$ of H^+ uptake. At light intensities between 14 and 20 μEin s^{-1} m^{-2} those thylakoids gave a large HEM-dependent H^+ uptake, indicative of lumenal H^+ deposition. At 14 μEin m^{-2} s^{-1} or below, the failure of HEM to stimulate H^+ uptake in the calmidazolium treatment suggests that the drug blocked H^+ deposition into the lumen. In comparison, the control membranes showed HEM stimulation of H^+ uptake at 6 and 10 μEin m^{-2} s^{-1}, consistent with H^+ deposition into the lumen at those low intensities and subsequent enhanced total H^+ uptake by the HEM amine.

Electron transport rates at 10, 14 and 50 μEin s^{-1} m^{-2}, measured in other thylakoid preparations, corresponded to 38, 50 and 100% of the maximum, uncoupled rates.

In the experiments of Fig. 3, the calmidazolium-treated thylakoids, in the absence of HEM, had a slightly lower H^+ uptake compared to the controls. The reason for this is not clear, but it cannot be due to a greater H^+ leak in the calmidazolium treatment. We measured the $t_{1/2}$ of the H^+ pump dark decay reaction in control and drug-treated membranes and found essentially the same half-times (10.6 ± 0.5 and 10.5 ± 0.5 s for the minus and plus 60 μM calmidazolium treatments, respectively). In any event, the effect is marginal.

The experiment shown in Fig. 3 was repeated on three different thylakoid preparations (different days as well) with consistent results. Namely, below the predicted maximum H^+ binding capacity of the sequestered domains, $\approx$ 120–140 nmol H^+ (mg Chl)$^{-1}$, and when electron transport capacity was limited by low light intensities, the calmidazolium treatment in the storage stage blocked amine-dependent extra H^+ uptake. Above a H^+ uptake level of $\approx$ 140 nmol H^+ (mg Chl)$^{-1}$ and when the electron transport capacity was near 50% of the maximum, the drug had little or no effect on the amine-dependent extra H^+ uptake.

Treatment of high-salt stored thylakoids with calmidazolium had no effect on H^+ uptake with or without the amine added (data not shown).

In similar experiments, with low-salt stored thylakoids under basal (−ADP) conditions, photoaffinity-bound chlorpromazine blocked more than 90% of the amine-(HEM) dependent H^+ uptake at the lowest light level (10 μEin s^{-1} m^{-2}) but had very little effect at the two higher light intensities (Table III A).

Those results are very similar to the data of Fig. 3 where calmidazolium was used, in that for H^+ uptake levels less than 140 nmol H^+ (mg Chl)$^{-1}$ with the light intensity sufficient to give less than 50% of the maximum electron transport rate, the drug blocked lumenal H^+ uptake. At light intensities giving 50% or more of the electron transport capacity (14 μEin s^{-1} m^{-2} in Table III) and H^+ uptake levels > 140 nmol H^+ (mg Chl)$^{-1}$, covalently bound chlorpromazine did not block the HEM-dependent (lumenal) H^+ uptake.

Table III. Effect of Covalently Bound Chlorpromazine on Basal H^+ Uptake (Steady Light)[a]

Treatments	Light intensity (μEin s^{-1} m^{-2})	Extent of H^+ uptake (nmo H^+/mg Chl)		Difference
		−HEM	+ HEM	
A. Low-salt storage				
1. − CPZ	10	137 ± 6	286 ± 10	149
	14	140 ± 4	381 ± 18	241
	50	172 ± 17	693 ± 66	521
2. + CPZ	10	134 ± 14	144 ± 6	10
	14	147 ± 10	374 ± 22	227
	50	167 ± 9	658 ± 31	491
B. High-salt storage				
1. − CPZ	10	162 ± 8	295 ± 17	133
	14	181 ± 12	395 ± 35	214
2. + CPZ	10	153 ± 14	285 ± 12	132
	14	171 ± 4	427 ± 18	256

[a] The procedures for UV treatment of low- and high-salt stored thylakoids and for the H^+ uptake assays were as described under Methods. The chlorpromazine concentration in the storage and UV treatment media was 10 μM. Thylakoids were suspended in the UV exposure medium at 33 μg Chl/ml and exposed to UV-A light for 10 min. The membranes were then pelleted and washed with low-salt or high-salt storage medium and resuspended in either medium at 3–4 mg Chl/ml. Each sample is the mean ± S.E. of three assays.

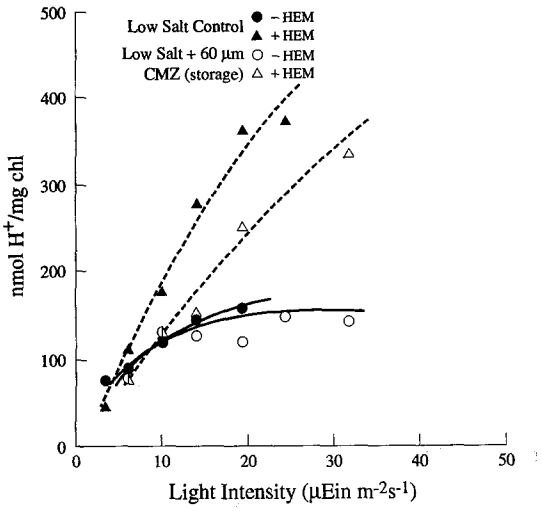

Fig. 3. Effect of calmidazolium on H^+ uptake with and without a permeable amine added. Total H^+ uptake was measured in low-salt stored thylakoids as described in Mthods. Calmidazolium (CMZ) at 60 μM was present only in the storage stage of one sample. In some measurements 0.5 m hydroxyethylmorpholine (HEM), pK_a 6.2, was added to the assay medium 3 min prior to illumination.

High-salt stored thylakoids did not show any effects of chlorpromazine photoaffinity binding at either the lowest or at the higher light intensity (Table IIIB).

DISCUSSION

The experiments reported here support the hypothesis that Ca^{++} ions, bound to the lumenal side of the CF_0H^+ channel, function in a gating mechanism to regulate H^+ flux across the inner boundary of the channel, *from either direction.* It seems of physiological importance that the closed-gate configuration is readily switched open, in part under control of the protonation state of the (presumed) carboxyl groups which we suggest provide the Ca^{++}-binding site for the gating structure. The dark, acid–base jump ATP formation, with lumenally loaded succinic acid as the main H^+ source, was more than 50% inhibited by photoaffinity-linked chlorpromazine when low-salt stored thylakoids were used in a pH 5.5 → 8.5 pH jump (Table II). We ascribe the inhibition as owing to the phenothiazine, having been bound to the Ca^{++}-occupied gating site, causing an increase in the Ca^{++}-binding affinity [a known effect of phenothiazines bound to proteins such as calmodulin (Massom *et al.*, 1990)], thus keeping the "gate" into the CF_0 closed.

The closed-gate condition could be readily converted to the open-gate response in the low-salt stored chlorpromazine and UV-treated membranes by lowering the acid stage pH to 4.5 (Fig. 1), or, while keeping the pH at 5.5, by washing the photoaffinity-treated low-salt stored thylakoids in the high-salt medium (Fig. 2). In the latter case the 100 mM K^+ may compete with the Ca^{++} for binding sites and in the former case the pH 4 conditions may be sufficiently acidic to permit H^+ ions to compete with the Ca^{++} ions; in either case the Ca^{++} could be displaced from the gating site by a monovalent ion, allowing the putative closed gating site to open. When no chlorpromazine was present, either pH 4 or pH 5.5 acid stage conditions gave a large succinic acid stimulation of ATP yield, consistent with an open CF_0 channel between the lumen and the CF_1. Those actions are consistent with the notion that the putative Ca^{++}-carboxyl gate structure is controlled by the protonation state of COO^- groups. Sufficiently acidic conditions are suggested to displace the bound Ca^{++}, allowing a structural change to the gate-open configuration.

Most if not all of the succinic acid-stimulated ATP formation for *either* low- or high-salt stored membranes is driven by H^+ ions released by the weak acid *in the lumen* as the thylakoids experience the alkaline shift (Uribe and Jagendorf, 1967). The lower ATP yield [$\approx$ 20 nmol (mg Chl)$^{-1}$], when HCl is used to acidify the thylakoids, is thought to be driven by the deprotonation of endogenous carboxyl groups. Our early studies have indicated that $-COO^-$ buffering groups occur both in the lumen and in the sequestered domains (Beard *et al.*, 1988; Beard and Dilley, 1988). It is expected that the endogenous sequestered domain carboxyl group buffering array, as well as COO^-–groups exposed in the lumen, would be largely protonated by the pH 4.0 conditions and partially protonated at pH 5.5. The magnitude of the domain COO^- buffering pool has been estimated to be about 120–150 nmol–COO^-/mg Chl (Beard *et al.*, 1988). The proportion of the acid–base jump ATP formation contributed by H^+ ions coming from the protonated carboxyl groups in the domains compared to –COOH groups in the lumen (when HCl rather than succinic acid is used for the acid stage) has not been precisely determined. However, it may be about one-half of the $\approx$ 20 nmol ATP (mg Chl)$^{-1}$, because photoaffinity-activated chlorpromazine inhibited about 50% of the acid–base jump ATP yield owing to acidification by HCl (data not shown). The portion

of acid–base jump ATP formation dependent on the membrane domain H^+ ions is expected to not be inhibited by chlorpromazine (treatment only in the storage stage). That conjecture is supported by the earlier observations that flash-driven, localized $\Delta\tilde{\mu}_{H^+}$ coupling was not inhibited by chlorpromazine (storage stage only) although postillumination ATP formation dependent on luminally located amine buffer was inhibited by the chlorpromazine treatment in low-salt stored thylakoids (Chiang and Dilley, 1988; Dilley and Chiang, 1989). In those experiments, high-salt stored membranes showed no influence of chlorpromazine on either ATP formation in the flash train or on postillumination ATP formation. Thus, the earlier data led to the hypothesis that a Ca^{++}-binding site was occupied by Ca^{++} in low- but not high-salt stored thylakoids, and that the binding site must be at the lumen side of the CF_0 H^+ channel (Chiang *et al.*, 1992; Dilley, 1991).

High-salt stored thylakoids, known from many other experiments to exhibit delocalized energy coupling (Beard and Dilley, 1986, 1988; Renganathan *et al.*, 1991, 1993) showed no chlorpromazine + UV inhibition in the pH 5.5 to 8.5 pH jump protocol, but when 1.5 mM $CaCl_2$ was present in the 100 mM KCl storage medium, the ATP yield was nearly as sensitive to the chlorpromazine + UV treatment as low-salt stored thylakoids (Table II). That is consistent with earlier observations of photophosphorylation showing that adding 1 mM Ca^{++} to the high-salt storage stage resulted in a return to the localized coupling response typical of low-salt stored thylakoids (Chiang and Dilley, 1987). The lower ATP yield in part 3 of Table II (high salt + 1.5 mM $CaCl_2$ in the storage phase) for the control treatment (– chlorpromazine) is not understood. However, the important point remains, namely that chlorpromazine caused a significant inhibition of the ATP formation, in contrast to the lack of effect in high-salt storage conditions with no added $CaCl_2$.

The gating action was also observed in the direction of membrane-sequestered domains into the lumen by measuring the effect of chlorpromazine or calmidazolium on total H^+ uptake. The amine-dependent component of H^+ uptake is a measure of lumenal H^+ accumulation (Avron, 1971; Nelson *et al.*, 1971; Pick and Avron, 1976) owing to the amine, present in the lumen, buffering a portion of the H^+ ions delivered there. As protons are taken up by the amine, the lumen pH does not go as acidic as it does in the absence of the amine and more protons can be pumped into the lumen. In this assay system photoaffinity-bound chlorpromazine (Table III) and a non-covalently bound Ca^{++} site probe, calmidazolium (Fig. 3), blocked most of the *amine-dependent* H^+ uptake *only* in low-salt stored membranes and only at low levels of H^+ uptake. The latter point suggests that above a certain level of (presumably) sequestered domain H^+ uptake, around 130–150 nmol $(mg\,Chl)^{-1}$, the H^+ ion activity is sufficient to cause gate opening and lumenal H^+ accumulation.

Rather than creating a permanent block, the Ca^{++}-binding site probes used here apparently tighten the affinity of the Ca^{++} bound to the putative gate carboxyl groups [cf. Massom *et al.* (1990) for evidence for this effect with calmodulin] of low-salt stored thylakoids, and more acidic conditions are needed to displace the bound Ca^{++}.

The sharp increase in the amine-dependent H^+ uptake between 10 and 14 $\mu Ein\,s^{-1}\,m^{-2}$ light intensity and the failure of chlorpromazine to block the effect at 14 $\mu Ein\,s^{-1}\,m^{-2}$ deserves further comment, as this may appear rather arbitrary. We think not, but emphasize the notion that a critical level of sequestered domain H^+ uptake occurs, near 140 nmol H^+ $(mg\,Chl)^{-1}$, which may provide sufficient "proton pressure" (acidity) in the domains to achieve the displacement of the putative bound Ca^{++}, even though the Ca^{++} binding is probably tighter in the presence of chlorpromazine. This type of effect is hinted at by the large difference in amine-induced extra H^+ uptake observed between 10 and 14 $\mu Ein\,s^{-1}\,m^{-2}$ in the absence of the drug; i.e., even with no drug there was a sharp increase in the HEM effect when the H^+ uptake was near 130 nmol H^+ $(mg\,Chl)^{-1}$ (Fig. 3). It seems reasonable to view this effect in terms of a saturable buffer capacity of (probably) $-COO^-$ groups in the sequestered domains, which, when reached, allows the pH of the domains to drop acidic enough to protonate the putative Ca^{++}-binding carboxyl groups. The proton buffering capacity of the domains has been estimated from other work as near 120–150 nmol H^+ $(mg\,Chl)^{-1}$ (Beard *et al.*, 1988; Renganathan *et al.*, 1991).

Under coupling conditions (note that the H^+ uptake measured here was in basal conditions), the utilization of the H^+ gradients accumulating in the sequestered domains by ATP formation apparently keeps the domain pH from dropping low enough to cause gate opening. That suggestion is supported by

other experiments showing localized energy coupling and very little effect of permeable amines on total H^+ uptake in low-salt stored membranes given either single-turnove flash (Chiang *et al.*, 1991) or continuous illumination (Renganathan *et al.*, 1991). We would predict (and our recent lumen pH measurements confirm, see below) that under such coupling conditions the *lumen pH* is considerably *above*, and the *sequestered domain* pH *below*, 5.7, with the external pH at 8. The latter prediction of the domain acidity being ≤ 5.7 is necessary to account for the thermodynamic requirements for energizing ATP formation, and the prediction that the lumen pH remains much higher than pH 5.7 accounts for the lack of significiant amine effects on total H^+ uptake in low-salt stored thylakoids under coupling conditions.

Those predictions concerning lumen pH were supported by lumen pH measurements with the fluorescent dye pyranine (Renganathan *et al.*, 1993) which showed that at an external pH of 8.0, under coupling conditions, the lumen pH in low-salt stored thylakoids was near 7.0. With high-salt stored membranes the lumen pH was less than 6.0 in both basal and coupled conditions. The pH needed to initiate gate opening, obviously considerably below pH 5.7, has not yet been established.

CONCLUDING REMARKS

These results provide additional support for and insight into the function of a Ca^{++}-regulated proton flux gating mechanism in thylakoid membranes. Carrying out the H^+ flux-driven ATP formation assays in dark conditions shows that the gating function is not dependent on electron transport or redox-turnover-dependent energization, events that were present in the initial experiments leading to the proposal (Chiang and Dilley, 1987). Further work is needed to clarify the molecular details of how Ca^{++} ions interact with the membrane components, probably the 8-kDa CF_0 (Chiang *et al.*, 1992), to provide the reversible gating function. Recent evidence using [$^{45}Ca^{++}$] as a probe for tight Ca^{++} binding to protein transferred from SDS-PAGE gels to nitrocellulose paper (Charuk *et al.*, 1990) indicates that a highly purified sample of the 8-kDa CF_0 protein shows Ca^{++} binding (Zakharov and Dilley, 1993).

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Science Foundation and the Department of Energy. The authors thank Ms. Janet Hollister for excellent help in the preparation of the manuscript.

REFERENCES

Allnutt, F. T. C., Dilley, R. A., and Kelly, T. (1989). *Photosynth. Res.* **20,** 161–172.
Avron, M. (1971). In *Second International Congress on Photosynthesis* (Forti, G., Avron, M., and Melandri, A., eds), Dr. W. Junk, The Hague, Vol. II, pp. 861–871.
Beard, W. A., and Dilley, R. A. (1986). *FEBS Lett.* **201,** 57–62.
Beard, W. A., and Dilley, R. A. (1988). *J. Bioenerg. Biomembr.* **20,** 129–154.
Beard, W. A., Chiang, G. G., and Dilley, R. A. (1988). *J. Biomembr.* **20,** 107–128.
Boyer, P. D., Chance, B., Ernster, L., Mitchell, P., Racker, E., and Slater, E. C. (1977). *Annnu. Rev. Biochem.* **46,** 955–1026.
Charuk, J. H. M., Pirraglia, C. A., and Reithmeier, R. A. F. (1990). *Anal. Biochem.* **188,** 123–131.
Chiang, G. G. (1989). Ph.D. Thesis, Purdue University, West Lafayette, Indiana.
Chiang, G. G., and Dilley, R. A. (1987). *Biochemistry* **26,** 4911–4916.
Chiang, G. G., and Dilley, R. A. (1988). In *Photosynthesis* (*Bruggs, W.*, (ed.) *Plant Biology Series*, Academic Press, New York, Vol. 8, pp. 437–455.
Chiang, G. G., Wooten, D. C. and Dilley, R. A. (1992). *Biochemistry* **31,** 5808–5819.
Demmig-Adams, B. (1990). *Biochim. Biophys. Acta* **1020,** 1–24.
Dilley, R. A. (1991). *Curr. Top. Bioenerg.* **16,** 265–318.
Dilley, R. A., and Chiang, G. G. (1989). *Ann. N.Y Acad. Sci.* **574,** 246–267.
Dilley, R. A., Theg, S. M., and Beard, W. A. (1987). *Annu. Rev. Plant Physiol.* **38,** 348–389.
Ferguson, S. J. (1985). *Biochim. Biophys. Acta* **811,** 47–95.
Good, N. E., Izawa, S., and Hind, G. (1966). *Curr. Top. Bioenerg.* **1,** 75–112.
Guffanti, A. A., and Krulwich, T. A. (1988). *J. Biol. Chem.* **263,** 14748–14752.
Haraux, F. (1985). *Physiol. Veg.* **23,** 397–410.
Horner, R. D., and Moudrianakis, E. N. (1983). *J. Biol. Chem.* **258,** 11643–11647.
Jagendorf, A. T., and Uribe, E. G. (1966). *Proc. Natl. Acad. Sci. USA* **55,** 170–177.
Luvisetto, S., Cola, C., Conover, T. E., and Azzone, G. F. (1990). *Biochim. Biophys. Acta* **1018,** 77–90.
Mandolino, G., De Santis, A., and Melandri, B. A. (1983). *Biochim. Biophys. Acta.* **723,** 428–439.
Massom, L., Lee, H., and Jarrett, H. W. (1990). *Biochemistry* **29,** 67–681.
Melandri, B. A., Venturoli, G., De Santis, A., and Baccarini-Melandri, A. (1980). *Biochim. Biophys. Acta* **592,** 38–53.
Ort, D. R., Dilley, R. A., and Good, N. E. (1976). *Biochim. Biophys. Acta* **449,** 108–124.
Pick, U., and Avron, M. (1976). *Eur. J. Biochem.* **70,** 569–576.
Pick, U., Weiss, M., and Rottenberg, H. (1987). *Biochemistry* **26,** 8295–8302.
Prozialeck, W. C., Cimino, M., and Weiss, B. (1981). *Mol. Pharmacol.* **19,** 264–269.
Renganathan, M., Pan, R.-S., Ewy, R. G., Theg, S. M., Allnutt, F. C. T., and Dilley, R. A. (1991). *Biochim. Biophys. Acta* **1059,** 16–27.

Renganathan, M., Pfündel, E., and Dilley, R. A. (1993). *Biochim. Biophys. Acta,* **1142,** 277–292.
Roberts, D. M., Lukas, T. J., and Watterson, D. M. (1986). *CRC Rev. Plant Sci.* **4,** 311–339.
Rottenberg, H. (1985). *Mod. Cell Biol.* **4,** 47–83.
Sigalat, C., Haraux, F., de Kouchkovsky, F., Hung, S. P. N., and de Kouchkovsky, Y. (1985) *Biochim. Biophys. Acta.* **809,** 403–413.
Uribe, E. G., and Jagendorf, A. T. (1967). *Plant Physiol.* **42,** 697–705.
Van Belle, H. (1981) *Cell Calcium* **2,** 483–494.
Van Walraven, H. S., Marvin, H. J. P., Koppenbal, E., and Kraayenhof, R. (1984). *Eur. J. Biochem.* **144,** 555–561.
Zakharov, S., and Dilley, R. A. (1993). *FEBS Lett.*, submitted.