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Thylakoid lumenal pH determination using a fluorescent dye: correlation of lumen pH and gating between localized and delocalized energy coupling

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Thylakoid lumen pH measurements were made using the fluorescent dye 8-hydroxypyrene 1,3,6-trisulfonic acid (HPTS; pyranine). The purpose was twofold: (1) to develop the method for use in the fluorescence ratio mode (permitting assays independent of lumenal dye concentration and lumen volume); (2) to use the technique to measure transmembrane ΔpH values in thylakoids predicted by previously used criteria to be energizing ATP formation with a delocalized or localized protonmotive force. The dye was readily loaded into thylakoids at pH 6 and the loading, washing and resuspension steps did not disrupt the localized or delocalized energy coupling patterns, tested by the effect of a permeable amine on the flash number required to initiate ATP formation as described in earlier work (Beard, W.A. and Dilley, R.A. (1988) J. Bioenerg. Biomembr. 20, 129). Light-dependent dye fluorescence changes were recorded in continuous illumination over 10–20 s with low- or high-salt-stored thylakoids under either coupled or basal conditions to assess the extent of the ΔpH developed across the membranes. Low-salt stored membranes – which were predicted by the criteria developed earlier to show localized $\Delta\tilde{\mu}_{H^+}$ energy coupling – gave a ΔpH below the thermodynamically predicted energetic requirement for ATP formation at external pH of 7.8 to 8.9. At an external pH of 7.8 or 8.0 the lumen pH under coupled conditions in the low-salt-stored thylakoids was about 6.8 to 7.0, respectively, (ΔpH $\approx$ 1.0 unit), and at pH 8.2 the ΔpH was about 1.4 units (lumen pH = 6.8). At pH 8.9 the lumen pH reached near 7.3 (ΔpH 1.6) under the coupled conditions for low-salt-stored thylakoids. Under basal conditions (no ADP) low-salt-stored membranes gave a ΔpH of 2.4 to 2.8 units, at moderate and high intensities, respectively, when the external pH was 8.9. The pH calibration is not sensitive enough at pH values less than about 6.0 to 6.3 to obtain accurate internal pH data in that range. For that reason, when the external pH was 7.8 to 8.0 all that can be said is that the basal ΔpH could be observed to be > 1.7 units. At an external pH of 8.9, high-salt-stored thylakoids – predicated to have a delocalized $\Delta\tilde{\mu}_{H^+}$ coupling mode – showed a ΔpH > 2.3 in all cases, coupled or basal, but in the coupled mode the ΔpH was near 2.3 at low light and near 2.7 at high light intensity. That is the expected result of the faster electron-proton transport at the high intensity. These lumen pH measurements support the concept developed with other techniques that thylakoids can maintain either localized or delocalized energy coupling $\Delta\tilde{\mu}_{H^+}$ gradients, and that in the localized energy coupling mode, the H^+ ions involved in driving ATP formation do not equilibrate with the lumen.

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Abbreviations used: ΔpH, pH of the assay medium minus that of the thylakoid lumen; DBMIB, dibromomethylisopropylbenzoquinone; DCMU, 3-(3,4 dichlorophenyl)-1,1-dimethylurea; DPX, *p*-xylene-bispyridinium bromide; ΔF_{450}, fluorescence emission changes at 511 nm induced by actinic light at 450 nm excitation; ΔF_{405}, fluorescence emission changes at 511 nm induced by actinic light at 405 nm excitation; HEM, hydroxyethylmorpholine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPTS, 8-hydroxypyrene 1,3,6-trisulfonic acid (pyranine); Mes, 2-[*N*-morpholino]ethanesulfonic acid; MV, methylviologen; Pyr, pyridine; Taps, *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

Chloroplast thylakoids stored in low-salt or high-salt suspension media show, respectively, a localized [1–7] or delocalized [5–11] proton gradient coupling to ATP formation. This concept has been well characterized using the permeable amine buffers pyridine and hydroxyethylmorpholine (HEM), in three types of experiment. The main experiment in this laboratory and that of Ort and colleagues involves observing the effect of an amine – located in the lumen – on the number of light flashes needed to reach the energization onset

[1,2,6,7,12], another protocol we have used utilized steady illumination conditions with the amine added at 40 s after turning on the light [13] and the third approach was to measure total H^+ accumulation with and without the amine present [13,14]. In all three experiments, theory and observation combined to give a clear pattern wherein low-salt-stored thylakoids in coupled conditions respond as though the H^+ gradient necessary to energize ATP did not equilibrate with the lumen (care was taken to show that the amines were reaching the lumen with similar kinetics in both types of thylakoid [13]), although under basal conditions those same thylakoids showed H^+ uptake into the lumen [13,14]. High-salt-stored thylakoids, in *either* coupled or basal conditions, responded as though the H^+ accumulation always equilibrated with the lumen.

Clear though the two coupling patterns appeared to be, the fact that most of the experiments depended on the action of amines in the lumen suggests that other types of experiments are called for which can test the localized-delocalized energy coupling pattern. One such approach is to more directly measure the lumen pH under the various states which can be generated with these thylakoids; i.e., basal and coupled conditions in the low- or high-salt-stored membranes. This work reports on the use of a pH-sensitive fluorescent dye, HPTS, also known as pyranine, loaded into the thylakoid lumen.

Other techniques for measuring lumen pH have been reported from time to time, and they all suffer from some difficulty or other. The distribution of a weak amine, say, [^{14}C]methylamine, has been the standard method [15,16], but it is complex and labor intensive to carry out (it usually involves centrifuging thylakoids to a pellet or through a silicon oil layer with unavoidable shading and some loss of energization), and it seems to result in a surprisingly large dark, non-energized state binding of the amine (enough to calculate out as a dark ΔpH near 0.6–0.8 even in the presence of nigericin [17]). Using 9-aminoacridine [18] brings in serious artifacts owing to binding of the cationic dye to the net negatively charged thylakoid [19,20], although it has been useful for relative ΔpH measurements [21]. Neutral red as a lumen pH probe, developed by Junge and colleagues [22], is heavily partitioned into the membrane phase, and it is not amenable to easy calibration as an accurate lumen pH indicator (cf. Refs. 23, 24 for discussion of these issues). The possibilities of ΔpH measurement using Tempamine as an electron spin resonance probe shows promise in the work of Tikhonov, Blumenfeld and colleagues (cf., Ref. 25 and Ref. 24 therein) but it is a highly specialized technique not readily available to many workers. Therefore, the development of a new class of pH (and other ion) fluorescent indicator dyes by Tsien and colleagues [26,27] was of interest to us. In this case we used 8-hydroxypyrene-1,3,6-trisulfonic acid, commonly known as pyranine, which proved to have properties suitable for lumen pH measurements.

One of the advantages of fluorescence detection is its inherent great sensitivity and another, for some dyes at least, is that detection of fluorescence at two wavelengths allows ratioing the ΔF and in that mode the change in the fluorescence ratio is independent of dye concentration and the volume of the compartment holding the dye [27]. These characteristics enormously simplify the calibration, data collection, and calculations correlating ΔF (ratio) with the lumen pH.

Methods

Chloroplast thylakoid isolation

Pea (*Pisum sativum,* cv. Little Marvel) plants were grown in a growth chamber at a light intensity of 450 μE m^{-2} s^{-1} with a 12 h light and 12 h dark cycle. The growth temperature was maintained at 18°C and 15°C during the light and dark cycles, respectively. Thylakoids were isolated from 14-d-old pea plants as described in Ref. 13 and stored either in 'low-salt' suspension medium containing 200 mM sucrose, 5 mM Hepes-NaOH (pH 7.5), 2 mM $MgCl_2$ and 0.5 mg ml^{-1} bovine serum albumin (BSA) or in 'high-salt' suspension medium in which the 200 mM sucrose was replaced by 100 mM KCl and 30 mM sucrose. Those media allow for the observation of localized or delocalized energy coupling, respectively [5,6]. Chlorophyll concentration was estimated by the method of Arnon [30] and thylakoids were usually stored at a Chl concentration of 2 to 4 mg ml^{-1}.

HPTS loading

HPTS was purchased from Kodak and loaded in the free acid fluorescent form. Chloroplast thylakoids do not have effective esterases to hydrolyze the permeant acetoxymethylester forms of cation sensitive dyes introduced by Tsien and colleagues [26,27] but as found by Bush and Jones for a variety of plant cells [30,31] the free acid forms of many of the dyes load satisfactorily (if somewhat slowly) at acidic pH values (pH 6.0 is sufficient in this case). Dye loading was initiated by adding 1 mg ml^{-1} of either low-salt- or high-salt-stored thylakoids to the dye-loading buffer consisting of 200 mM sorbitol, 20 mM Mes-KOH (pH 6.0), 3 mM $MgCl_2$, 20 mM KCl and 0.5 to 1 mM HPTS. After incubating at 15°C for 30 min this mixture was stored on ice for the rest of the experiment (up to 4 h). Once dye is loaded into the thylakoid lumen, by simply changing the external buffer pH to alkaline conditions traps the dye in the lumen. 100 μl Aliquots were taken from this mixture and diluted to 1 ml with washing buffer, consisting of 200 mM sorbitol, 20 mM Taps-KOH (pH 8.9), 3 mM $MgCl_2$ and 20 mM KCl. This diluted mixture

was centrifuged in a microfuge for one min and the pellet was suspended in fresh buffer, and centrifuged again. The final pellet was suspended in 100 μl of the washing buffer and used for not more than one assay.

pH sensitivity of HPTS fluorescence intensity

Fluorescence emission at 511 nm was measured in an SLM 8000 spectrofluorimeter equipped with a DMX 1000 unit using a 1 cm pathlength cuvette. The entrance slit bandpass into the excitation monochromator was 8 nm and the exit bandpass was 2 nm. For the emission monochromator the entrance and exit bandpasses were 8 and 16 nm, respectively. The assay buffers for pH calibration consisted of 20 mM Taps-KOH buffer for pH 9.0 to 8.0, 20 mM Hepes-KOH for pH 7.5 and 7.0, or 20 mM Mes-KOH buffer for pH 6.5 to 5.5, and in all cases 200 mM sorbitol, 3 mM $MgCl_2$ and 20 mM KCl.

pH calibration of dye-loaded thylakoids

The in vivo sensitivity of the HPTS was established by recording the excitation spectra of the dye-loaded thylakoids from 300 to 490 nm at different pH values with the emission set at 511 nm. The pH calibration buffers were as mentioned above except for additions such as 0.1 mM methylviologen, 0.2 mM ADP, 5 mM KH_2PO_4, 100 nm nonactin, hexokinase at 120 units ml^{-1} (highly purified hexokinase from Sigma, catalog No. H-4502, was used to avoid any coloration in the assay mixture which could possibly interfere in the fluorescence measurements) and 20 mM glucose to keep the calibration similar to the lumenal pH assay conditions. Nigericin at 1 μM and the monovalent ionophore nonactin were added to equilibrate the proton concentration across the thylakoid membranes. Thylakoids not loaded with dye show significant background fluorescence which is somewhat pH-dependent and a pH calibration curve was obtained after correcting for this fluorescence at each pH.

pH measurement of the thylakoid lumen

The lumenal pH assay buffer consisted of dye-loaded thylakoids at 10 μg Chl ml^{-1}, 200 mM sorbitol (except where noted, when the sorbitol was omitted), 20 mM Taps-KOH (pH 8.9) (or other buffers as specified in the figures), 3 mM $MgCl_2$, 20 mM KCl, 0.1 mM methylviologen, 0.2 mM ADP (or no ADP for basal conditions), 5 mM KH_2PO_4, 100 nm nonactin, hexokinase at 120 units ml^{-1} and 20 mM glucose. Hexokinase and glucose were included as an ADP-regenerating system to keep the ADP at a steady concentration in the assay mixture, although experiments done without those additions gave identical results. Two ml of this assay mixture was placed in a cuvette, magnetically stirred and temperature controlled at 10°C, and the dye fluorescence emission at 511 nm was monitored using 450 and 405 nm excitations.

pH changes were initiated by giving $CuSO_4$ filtered white actinic illumination focused on a Corning CS 2–64 red filter placed in front of a flexible fiber-optic light guide. The light guide was coupled to a Plexiglas light randomizer which was snugly fit into the window of the cuvette holder and placed against the cuvette so as to illuminate the entire cuvette contents. The emission monochromator was protected from the red actinic light by a Corning CS 4–96 blue filter.

Flash-induced ATP formation using the luciferin-luciferase enzyme system

Beard and Dilley [5,6] have explained in detail the assay for ATP formation driven by single-turnover flashes using the luciferin-luciferase enzyme complex. Single-turnover flashes ($\approx$ 10 μs at half intensity) from a xenon lamp (EG&G, FX-200) were transmitted by a Schott RG 630 filter and delivered via a flexible light guide to a light-protected cuvette with the temperature maintained at 10°C. ATP-induced luciferin-luciferase chemiluminescence at 560 nm was detected by a photomultiplier tube (EMI 9558Q) protected from the red actinic light by a Corning 4–96 filter. The resulting signal was amplified through a Tektronix 5A22N differential amplifier and recorded on a strip chart recorder.

ATP formation assay was initiated by adding 15 μg Chl ml^{-1} at $t = 0$ into 800 μl of assay buffer mixture containing 50 mM Tricine-KOH (pH 8.0), (or pH 8.9 for some experiments), 10 mM sorbitol, 3 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM DTT, 400 nM nonactin, 0.1 mM ADP (Dowex purified), 5 μM diadenosine pentaphosphate (Sigma), an inhibitor of adenylate kinase and 0.1 mM methyl viologen. At $t = 2.5$ min, 10 μl of luciferin-luciferase reagent (one bottle of the LKB ATP monitoring reagent was dissolved in 1.5 ml of cold distilled water and aliquots frozen until use) was added and at $t = 3.5$ min ATP formation was initiated by a sequence of 150 flashes at 5 Hz. To test the permeable buffer effect on the ATP onset lag, 5 mM pyridine (pK_a 5.4) or 0.5 mM hydroxyethylmorpholine (pK_a 6.3) was added to the assay buffer and thylakoids were incubated in this assay mixture for 3.5 min for the complete equilibration of the amine across the thylakoid membrane prior to the train of 150 flashes. At the end of each assay the signal was calibrated by addition of standard ATP.

Steady illumination ATP formation

ATP formation by steady illumination was assayed by detecting luciferin-luciferase luminescence at 10°C using 0.8 ml reaction mixture which contained 2 μg Chl ml^{-1}, 200 mM sorbitol, 20 mM Taps-KOH (pH 8.9), 3 mM $MgCl_2$, 20 mM KCl, 5 mM KH_2PO_4, 5 mM DTT, 0.1 mM MV, 5 mM A_2P_5, 0.1 mM ADP (Dowex

purified), 100 nM nonactin and 10 μl of the luciferin-luciferase complex. Thylakoids were used at 2 μg Chl ml^{-1} to avoid excessive ATP formation during the 1 min light period, which would desensitize the luciferase. White actinic light from a 500 W projection bulb was passed through a $CuSO_4$ solution, one Corning CS 2–64 red filter and focused through a light guide leading to the cuvette. Light intensity used for ATP formation was 24 μmol m^{-2} s^{-1} at the cuvette surface. The permeable amine HEM was injected into the ATP formation mixture 24 s after the onset of ATP formation.

Results

pH sensitivity of the excitation spectra of HPTS

The pH dependence of the HPTS excitation spectra, in buffer with no chloroplast material present, was recorded between 300 to 550 nm and is shown in Fig. 1A. When excited at 450 nm, fluorescence at 511 nm is most intense at pH 9.0 and it decreases with pH. With excitations at 405 and 369 nm, fluorescence is lowest at pH 9.0 and it increases with decreasing pH. By selecting the ratio of 450 over 405 nm this dye shows a 4-fold decrease in fluorescence intensity from pH 9.0 to pH 6.0 (inset of Fig. 1A). The pK_a of HPTS, reported to be 7.3 [28], could be calculated from Fig. 1B which shows the pH response of 450 and 405 nm excited fluorescence emission at 511 nm. The crossover point of the 450 and 405 curves is at the midpoint of their pH response, and it agrees with the published pK_a.

pH sensitivity of HPTS-loaded thylakoids

Thylakoids not loaded with the dye – excited at 450 or 405 nm – emit a weak but significant endogenous fluorescence at 511 nm which is about 6% at pH 9.0 and 10% at pH 5.5, of the total fluorescence with HPTS present using either 450 or 405 nm excitation (Fig. 4). The endogenous fluorescence at each pH was subtracted from the total fluorescence of the dye-loaded thylakoids to obtain the pH calibration (and in all ΔpH assay calculations). The corrected excitation spectra of the HPTS-loaded thylakoids from pH 9.0 to 5.5 are shown in Fig. 2 in the presence of 1 mM nigericin and 100 nM nonactin to equilibrate the proton concentration. Fig. 2 shows that 450 nm excited fluorescence at pH 9.0 is more or less equal to the 405 nm excited fluorescence at pH 5.5, whereas the pH-dependent HPTS curves in Fig. 1 show that the 450 nm excited fluorescence at pH 9.0 is 1.6-fold higher than the 405 nm excited fluorescence at pH 5.5. One reason for this is that chlorophyll absorption is greater at 450 than at 405 nm. The difference in the spectral shape accounts for the 0.3 pH unit shift to the alkaline side in the calibration curve of HPTS-loaded thylakoids compared to the control HPTS calibration curve (Fig. 3).

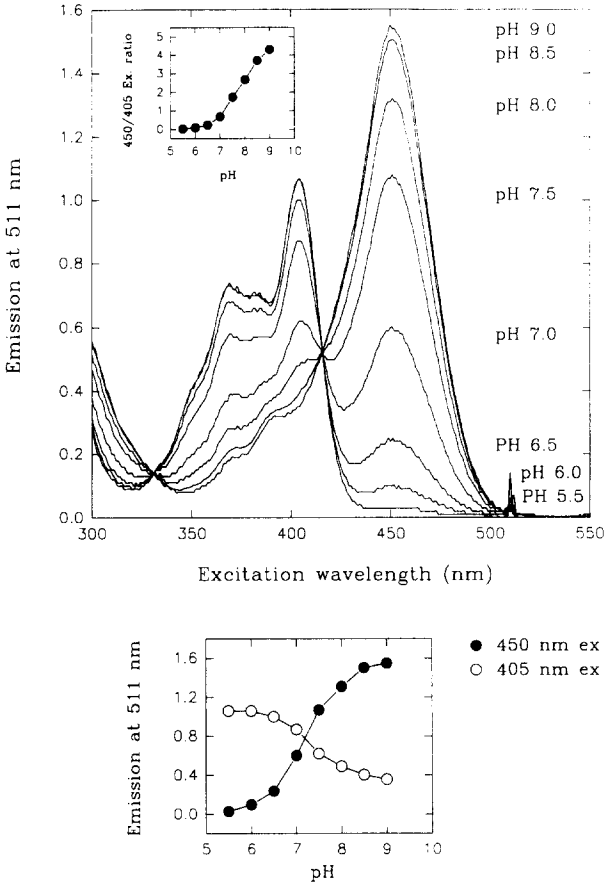

Fig. 1. (A) Excitation spectra of 1 μM HPTS recorded in a pH range of 5.5 to 9.0. The assay buffers for pH calibration consisted of 20 mM Taps-KOH buffer for pH 9.0 to 8.0, or 20 mM Hepes-KOH for pH 7.5 and 7.0, or 20 mM Mes-KOH buffer for pH 6.5 to 5.5 and 200 mM sorbitol, 3 mM $MgCl_2$ and 20 mM KCl. The excitation spectra were recorded between 300 and 550 nm with the emission monitored at 511 nm in a SLM 8000 spectrofluorophotometer equipped with DMX 1000 unit as described in under Methods. Inset is the pH response of the ratio of 450 nm/405 nm excitation. (B) Excitation intensity of 511 nm emission at 405 and 450 nm at various pH values.

The light-induced fluorescence changes of the HPTS-loaded thylakoids and their corresponding lumenal pH changes are estimated from the pH calibration of HPTS-loaded thylakoids and not from the HPTS-only calibration, hence the 0.3 pH unit alkaline shift does not interfere with the pH estimation.

Effect of HPTS loading and thylakoid washing on energy coupling

After the acidic pH dye loading step, free dye from the loading buffer was removed by two washes of the thylakoids with the higher pH assay buffer. Centrifuging and resuspending thylakoids can decrease the efficiency of ATP formation, so it was necessary to test whether the two washing steps, or the dye loading itself produced significant deleterious effects. In particular it was important to test whether the protocol caused low-salt-stored thylakoids – normally observed to have

a localized coupling mode – to respond in a delocalized mode. The assays we routinely have used to demonstrate localized or delocalized energy coupling are: (A) the effect of a permeable amine (at concentrations less than those giving uncoupling) on the number of single-turnover flashes needed to reach the energetic threshold for ATP formation [6,7], and (B) the effect of adding the amine to a thylakoid sample roughly 40 s after the beginning of steady illumination [13]. The latter protocol is more similar to the light-dark cycles we will use in the following HPTS fluorescence changes, but we tested both protocols anyway.

By both of these criteria the ΔpH assay buffer used and the HPTS loading and washing steps were amenable to low-salt-stored thylakoids maintaining localized energy coupling characteristics, although the treated samples were less efficient in ATP formation. Using the ATP formation onset lag criterion, one compares the onset flash number without the amine to that with the amine present (prior to the beginning of the flash train [6,7]). Localized coupling is suggested to occur when there is little or no effect of the amine on the onset lag, whereas delocalized coupling is indicated when the amine causes an increase in the onset lag of 15 or so flashes [6,7]. Table I compares the usual way we have carried out the phosphorylation assay (pH 8.0 Tricine media, no washing steps) with the pH 8.9 Taps buffer medium used in the ΔpH experiment. Low-salt-stored thylakoids, washed twice and assayed at pH 8.9 with or without HPTS loading, showed only a slight effect of the amine on the ATP formation onset lags compared to the pH 8.0 control treatments (HEM-induced lags of 2/4 versus −2/4, respectively, cf. the legend of Table I for the explanation of the lag parameters). The lag values of −2/4 indicate little effect of the treatments on proton gradient localization, but the lower ATP yield per flash suggests that some effect, perhaps on the coupling factor, follows the treatment. The high-salt-stored treatment gave the expected much longer onset lag when the amine was present, typical values being 15/20 flashes for the pH 8.0 condition or 10/10 for the pH 8.9 after HPTS loading.

For the continuous illumination experiments the criterion for assessing localized or delocalized coupling is whether the amine addition during steady phosphorylation causes a transient delay in the rate of ATP formation (see Fig. 1 of Ref. 13 for examples). Table II shows that HPTS loading and pH 8.9 assay conditions

TABLE I

Effect of 1 mM HPTS loading and washing on energy coupling modes of thylakoids

Control thylakoids (thylakoids not loaded with HPTS and not washed), thylakoids not loaded with 1 mM HPTS but washed, thylakoids loaded with 1 mM HPTS and washed twice with dye washing buffer, were used to study the effect of 0.5 mM HEM on the flash onset lag. ATP formation onset lag was measured as mentioned in Methods using 5 Hz flashes and 15 mg Chl ml^{-1} using 200 mM sorbitol, 20 mM Taps-KOH (pH 8.9), 3 mM $MgCl_2$, 20 mM KCl, 5 mM KH_2PO_4, 5 mM DTT, 0.1 mM methylviologen, 5 μM diadenosine pentaphosphate, 0.1 mM ADP (Dowex purified), 400 nm nonactin and 10 μl of luciferin-luciferase complex (from a stock of 1.5 ml LKB vial) HEM was added to the reaction mixture 1 min prior to turning on the flash train. Standard deviation for the onset lags were generally calculated to be ± 1 or 2 flashes.

Thylakoid storage	1 mM HPTS loaded	Washing	HEM	Flash lag	(+)-(−) HEM	Flash yield nmoles ATP mg Chl^{-1} $flash^{-1}$
A. pH 8.0 assay						
LS	no	no	no	21/28 [a]		0.71 ± 0.12
LS	no	no	yes	23/30	2/2	0.77 ± 0.06
HS	no	no	no	28/33		0.51 ± 0.01
HS	no	no	yes	43/53	15/20	0.45 ± 0.01
B. pH 8.9 assay						
LS	no	no	no	24/28 [a]		0.62 ± 0.12
LS	no	no	yes	26/32	2/4	0.57 ± 0.06
HS	no	no	no	26/35		0.53 ± 0.01
HS	no	no	yes	38/60	12/25	0.46 ± 0.05
LS	no	yes	no	58/72		0.14 ± 0.01
LS	no	yes	yes	56/76	−2/4	0.13 ± 0.01
LS	yes	yes	no	56/78		0.18 ± 0.01
LS	yes	yes	yes	58/78	2/0	0.11 ± 0.04
HS	no	yes	no	62/72		0.20 ± 0.02
HS	no	yes	yes	72/82	10/10	0.14 ± 0.05
HS	yes	yes	no	73/86		0.12 ± 0.02
HS	yes	yes	yes	86/96	13/10	0.10 ± 0.01

[a] The first of the two numbers listed as the flash lag (21/28, for example) is the flash number giving the first detectable rise in the ATP signal and the second is the flash number at the intersection of the horizontal line connecting the bottom of the flash signal train before ATP formation begins with the line formed by connecting the bottom of the flashes in the rising signal (cf. Ref. 14).

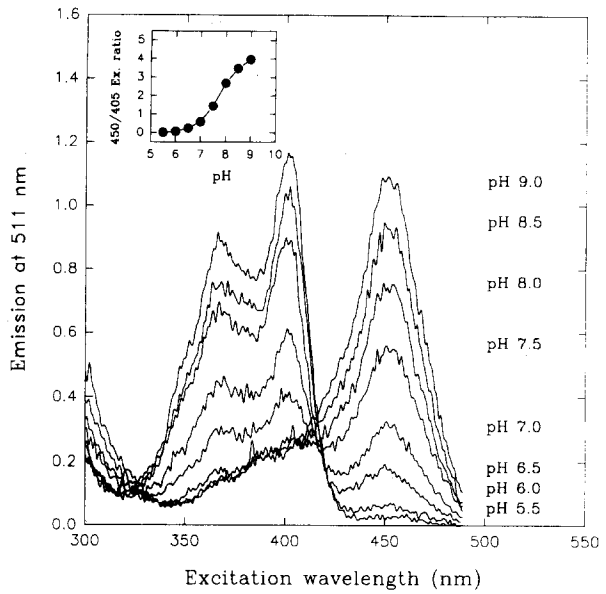

Fig. 2. Excitation spectra of HPTS-loaded thylakoids recorded in a pH range of 5.5 to 9.0. Assay conditions were similar to those in Fig. 1A using 1 mM HPTS except that in addition it contained 0.1 mM methylviologen, 0.2 mM ADP, 5 mM KH_2PO_4, 100 nM nonactin, hexokinase at 120 units ml^{-1} (highly purified hexokinase from Sigma, catalog No. H-4502, was used to avoid any coloration in the assay mixture which could interfere in the fluorescence measurements) 20 mM glucose and 2.5 μM nigericin. Since the emission was monitored at 511 nm, excitation spectra were recorded from 300 to 490 nm to avoid the scattering of excitation light by thylakoids into the emission monochromator. Inset. 450/405 excitation ratio of HPTS loaded thylakoids response to pH. 450/405 excitation ratio was calculated from the respective spectrum. This curve is virtually identical to the curve shown in the inset of Fig. 1.

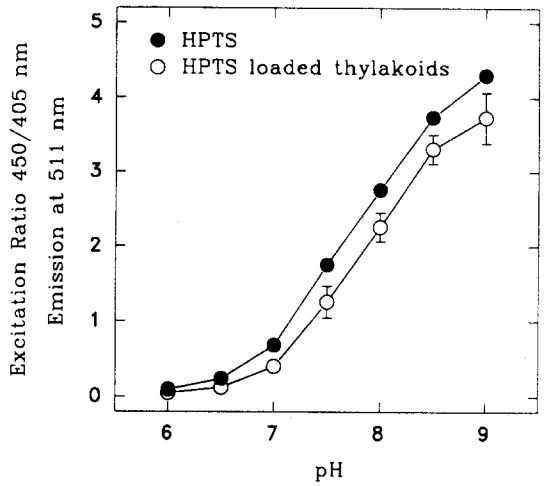

Fig. 3. Fluorescence ratio of HPTS versus pH without thylakoids present and HPTS loaded into thylakoids. See the methods section for details. The assay conditions for HPTS loaded thylakoids were the same as in Fig. 1A. The data for HPTS-loaded thylakoids represent the average of four low-salt-stored thylakoid and four high-salt-stored thylakoid preparations. The pH response of low-salt and high-salt stored thylakoids was similar.

had no deleterious effect on the ATP formation rate, although washing thylakoids twice significantly reduced the rate with both low and high-salt-stored membranes. The amine-induced delay was much larger in the high-salt-stored thylakoids, near 60 nmol/mg Chl compared to 16–26 nmol/mg Chl for the low-salt-stored samples, and neither HPTS nor washing altered that pattern significantly. Those results are similar to the earlier results using pH 8.0 assay conditions [13] and are consistent with the localized (low-salt) and delocalized (high-salt) coupling assignments.

We conclude that the HPTS loading and washing steps and the pH 8.9 assay conditions with low-salt-stored thylakoids did not cause $\Delta\tilde{\mu}_{H^+}$ delocalization, whereas in the treated high-salt-stored thylakoids the

TABLE II

Effect of 0.5 mM HEM on HPTS loaded low-salt and high-salt-stored thylakoids in continuous illumination ATP formation

Control thylakoids (thylakoids not loaded with HPTS and not washed), thylakoids loaded with 1 mM HPTS but not washed, thylakoids not loaded with HPTS but washed, and thylakoids loaded with 1 mM HPTS and washed twice with dye washing buffer, were used to study the effect of 0.5 mM HEM in H_2O to MV-mediated, steady-light-induced, ATP formation. ATP formation was assayed by detecting luciferin-luciferase luminescence at 10°C using 0.8 ml reaction mixture which contained 2 μg Chl ml^{-1}, 200 mM sorbitol, 20 mM Taps-KOH (pH 8.9), 3 mM $MgCl_2$, 20 mM KCl, 5 mM KH_2PO_4, 5 mM DTT, 0.1 mM methyl viologen, 5 μM diadenosine pentaphosphate, 0.1 mM ADP (Dowex purified), 100 nM nonactin and 10 μl of luciferin-luciferase complex (from a stock of 1.5 ml LKB vial) Light intensity used for ATP formation was 24 μmol $m^{-2}s^{-1}$. The permeable amine HEM was injected into the ATP formation mixture 40 s after the onset of ATP formation.

Thylakoid storage	0.5 mM HPTS loaded	Washing	Delay in ATP formation due to 0.5 mM HEM addition. nmol ATP $(mg Chl)^{-1}$	Rate of ATP formation μmol ATP $(mg Chl\, h)^{-1}$
LS	no	no	16±1	60±5
LS	yes	no	16±2	60±4
LS	no	yes	16±2	30±2
LS	yes	yes	22±4	36±9
HS	no	no	55±4	67±6
HS	yes	no	60±8	64±7
HS	no	yes	63±5	30±2
HS	yes	yes	57±3	30±8

$\Delta\tilde{\mu}_{H^+}$ was delocalized just as in the previous work [6,7].

Light-induced HPTS fluorescence changes

A common practice in using the cation-sensitive dyes is to load the permeable ester (non-fluorescence) form of the dye and depend on cellular or organellar esterases to hydrolyze the esters and produce the fluorescent, anionic form [26,27]. However, the thylakoid lumen does not contain esterase enzymes necessary to hydrolyze the ester bonds, so we must resort to loading the free acid form. At pH near 5.7 to 6.0, the HPTS loads into the lumen (cf., Methods) and by washing the acid-loaded thylakoids twice with high pH (8.9) assay medium we can remove most of the external dye and trap the anionic form in the lumen. Evidence for lumen loading is seen in Fig. 4, showing actinic light-dependent fluorescence changes at 511 nm (elicited by 450 and 405 excitation) expected for the dye experiencing an acidic shift. Nigericin at 5 μM completely blocked the fluorescence changes (Fig. 4, bottom) as did 5 μM DCMU (data not shown). The HPTS light-dependent fluorescence signal was not observed when non-loaded (low-salt-stored) thylakoids were suspended in the pH 8.9 assay medium and supplemented with 50 nM HPTS. The HPTS would remain in the external phase under those conditions, hence this control shows that the fluorescence changes are not caused by external phase HPTS interacting with the energized membranes (data not shown). The above data indicate that the light-dependent fluorescence signal (Fig. 4) is a response to lumenal acidification and not a function of the electron transport activity (which would continue in the presence of nigericin) or HPTS binding at the external surface. Light-induced fluorescence changes at both 450 and 405 nm excitation reached a steady state level by 5 s and when the actinic light was turned off the signal recovered to the original level. The $t_{1/2}$ for these fluorescence changes at 450 and 405 nm closely matches the $t_{1/2}$ of proton uptake and decay kinetics. A second cycle of actinic light showed a similar response indicating that acidification of the lumen during the first cycle of actinic illumination did not induce the dye to leak out (that would eventually occur owing to the acidic conditions in the lumen).

Fig. 4B shows the endogenous or background 511 nm fluorescence signal of thylakoids not loaded with HPTS. The background 511 nm fluorescence of thylakoids in the absence of HPTS, excited by 450 nm light, was less than 10% the usual intensity observed with HPTS-loaded thylakoids, but of more importance than that, there was no change in the background fluorescence when actinic light activated electron and H^+ transport. That result simplifies the calculation of the corrected (see below) ratio of HPTS fluorescence excited by 450 and 405 nm light.

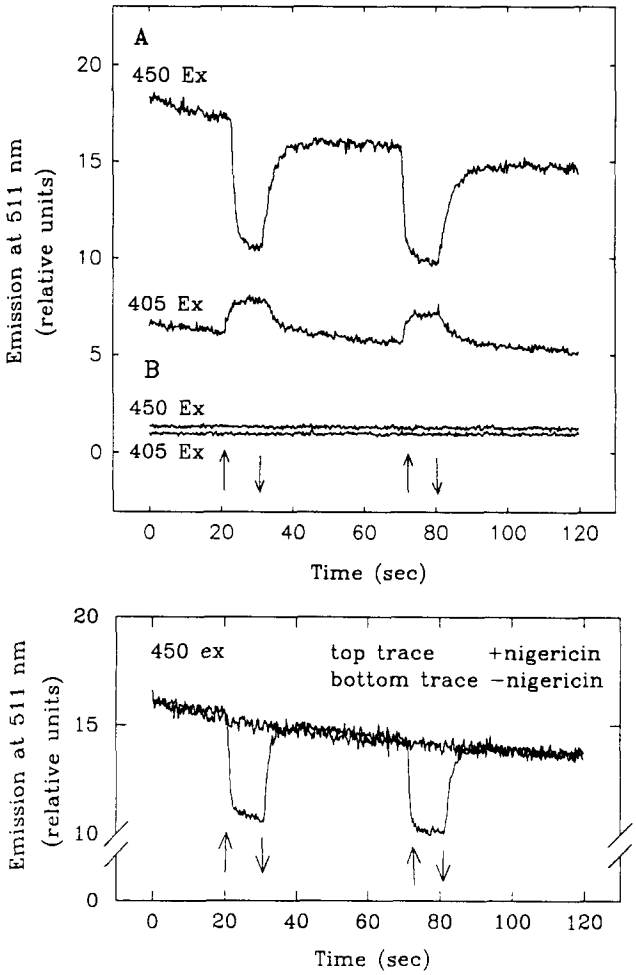

Fig. 4. (A) Light-induced fluorescence changes in HPTS-loaded thylakoids. The assay buffer consisted of 10 μg Chl ml^{-1} HPTS-loaded thylakoids, 200 mM sorbitol, 20 mM Taps-KOH (pH 8.9), 3 mM $MgCl_2$, 20 mM KCl, 0.1 mM methylviologen, 0.1 mM ADP (or no ADP for basal conditions), 5 mM KH_2PO_4, 100 nM nonactin, hexokinase at 120 units ml^{-1} and 20 mM glucose. Other conditions were as in Fig. 2. 2-ml aliquots of the above assay mixture were placed in a cuvette which was magnetically stirred and temperature controlled at 10°C. The upper trace represents time-dependent 511 nm fluorescence emission with 450 nm excitation. Lower trace represents time-dependent 511 nm fluorescence emission with 405 nm excitation. Upward arrows indicate actinic light on and downward light off. The actinic light was defined by a Corning CS 2–64 filter and the intensity was 24 μE m^{-2}s^{-1}. The emission monochromator was protected from the red light by a Corning CS 4–96 filter. (B) Background fluorescence of thylakoids not loaded with HPTS. Assay conditions were same as in (A). The upper trace is the time-dependent 511 nm fluorescence emission with 450 excitation, and the lower trace is the time-dependent 511 nm fluorescence emission with 405 excitation. *Bottom.* HPTS fluorescence excited by 450 nm light as in the top part of A above, but with or without 5 μM nigericin, as indicated.

Using the fluorescence ratio method for ΔpH determination

The advantage of dye signals from compounds such as HPTS as a means of estimating ion concentration is that the fluorescence change of the ratio (F_{450}/F_{405}) induced by changing ion concentration is independent

of dye concentration or volume of the organelle containing the dye as shown in the development of these methods by Tsien and colleagues [26,32]. The point is readily understood by considering that the pH-dependent ratio signal is calculated with dye concentration in both the numerator and the denominator of the ratio, thus the concentration term cancels (see Appendix for details). Fig. 5 shows a pH titration of the ratio measurements for HPTS-loaded thylakoids with either 0.5 or 1 mM HPTS used in the loading. Obviously, the amount of HPTS in the lumen could not, in both cases have been the same yet the plots of the ratio F_{450}/F_{405} vs. pH overlay one another perfectly, showing that the calibration curve is independent of dye concentration. This point was shown also by an experiment where the dye loading and the light-dependent fluorescence signal was assayed in either 200 mM sorbitol (the usual method) or no sorbitol added to the loading and assay buffers (Table III). There was no significant difference in the calculated ΔpH for either basal or coupled conditions in the lower tonicity conditions compared to the normal conditions using 200 mM sorbitol (see below, where Fig. 8 is presented and Table III is described further).

Another point needing clarification is the effect of dye present outside the thylakoid lumen (i.e., in a constant pH 8.9 condition) during the light on-off cycles. The Appendix shows that with no pH changes in the external medium and external dye remaining constant the fluorescence contribution of any external dye cancels out in the method. Keeping the illumination time short ($\approx$ 10 s) minimizes the amount of dye leaking out of the lumen. This was also verified using

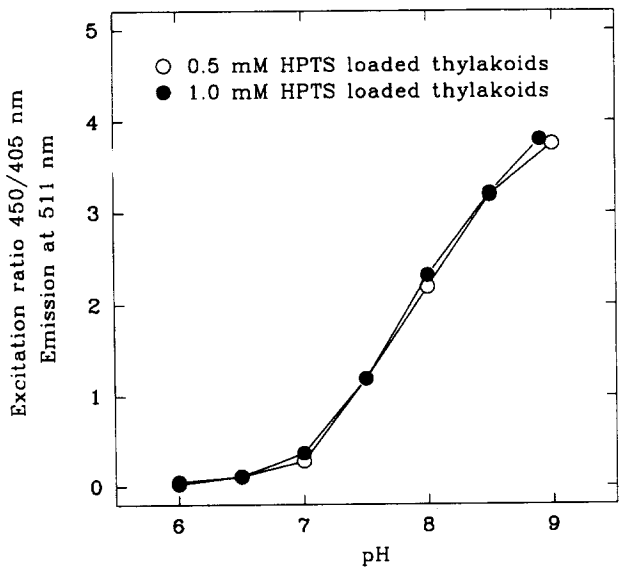

Fig. 5. Two independent calibration curves of the fluorescence ratio with 450 and 405 nm excitation and 511 nm emission. Conditions were similar to those of Fig. 3. The indicated HPTS concentrations were used for loading.

TABLE III

Effect of varying the dye loading and assay osmoticum on basal and coupled ΔpH values determined by HPTS

Low-salt-stored thylakoids were loaded as described in the Methods section in a medium containing 1 mM HPTS, either with no added sorbitol or with 200 mM sorbitol and assayed for changes of its 511 nm fluorescence excited at 450 and 405 nm to determine the ΔpH. The assay conditions were similar to those used in Fig. 4 except that the pH was 8.2.

Loading osmoticum	Assay osmoticum	Coupled ΔpH	Basal ΔpH
0 mM sorbitol	0 mM sorbitol	1.11 ± 0.13 n = 10	> 2.2 [a]
200 mM sorbitol	200 mM sorbitol	1.04 ± 0.10 n = 12	> 2.2
200 mM sorbitol	0 mM sorbitol	1.19 ± 0.01 n = 8	> 2.2

[a] Because the pH calibration curve of HPTS becomes very flat in the region of pH 6.0, it is not possible to determine precisely the lumen pH in the region below pH 6.0.

an externally located fluorescence quencher, and data will be presented below supporting the point that externally located dye does not interfere with the lumen pH determination using the present protocol.

Estimation of lumenal pH in various energy coupling modes

Lumenal pH was estimated by determining the ratio of $\Delta F_{450}/\Delta F_{405}$ in low-salt and high-salt-stored thylakoids under basal and coupled conditions. The issue here is to compare the measured ΔpH between the lumen and the external medium (it is assumed that the lumenal dye senses the bulk phase pH, and not that of the membrane domains), with the calculated ΔG_{ATP} needed to drive ATP formation. In the Discussion section this comparison will be developed. Suffice it here to say that thylakoids have been reported to develop a ΔpH near 2.5 to 3.0 units (using the distribution of an amine method) under basal conditions [15,18,20,33] when the external pH was in the range of pH 8.0 to 9.0. Does the HPTS probe method give comparable ΔpH data? The answer is yes, as seen in Fig. 6 for high-salt-stored thylakoids under basal conditions, which gave a ΔpH near 2.90 ± 0.03 units, when the external pH was 8.90. Those thylakoids under coupled conditions gave a ΔpH of 2.30 ± 0.09. Low-salt-stored thylakoids developed ΔpH values of 2.40 ± 0.14 and 1.60 ± 0.03 units for basal and coupled conditions, respectively. The data of Fig. 6 are combined results from twenty measurements using five different thylakoid preparations, all with a light intensity of 24 $\mu E\ m^{-2}\ s^{-1}$, and with glucose and hexokinase present to recycle the ATP formed back to ADP. The reason for including glucose and hexokinase was to keep the conditions of low ATP in these steady illumination experiments similar to the conditions used in the earlier work with the luciferin-luciferase system as the ATP detection method [6,12,14]. However, recycling

the ATP was not necessary for maintaining the ADP supply in the present experiments, because only 10% or less of the ADP was phosphorylated under our conditions. For comparison of the lumenal pH attained with higher ATP concentration, and, therefore, at a much higher ΔG_{ATP} against which the $\Delta\tilde{\mu}_{H^+}$ had to work to drive ATP formation, we did experiments with no hexokinase added. The results were the same, within experimental error, with or without hexokinase, with the ΔpH being near 1.6 for coupled conditions with the external pH 8.9 condition using low-salt-stored thylakoids and ΔpH values near 2.3 for high-salt-stored thylakoids (data not shown).

ΔpH determination at different light intensities

Increasing the incident light intensity on the cuvette for the ΔpH measurements from 24 μE $m^{-2}s^{-1}$ (the intensity used in the experiments shown in Fig. 6 and Table II) to 110 μE $m^{-2}s^{-1}$ revealed a progressive increase in the ΔpH for the low-salt basal and high-salt coupled cases, but with little or no change in the ΔpH for the low salt coupled and high salt basal cases (Fig. 7). The numbers above the three groups of experiments in Fig. 7 are the ATP formation rates determined in parallel on thylakoids used for the ΔpH experiments. The rate of ATP formation was saturated at the intermediate intensity, but as expected, the ΔpH achieved for the low-salt basal and high-salt coupled cases increased at the highest intensity. It should be noted that

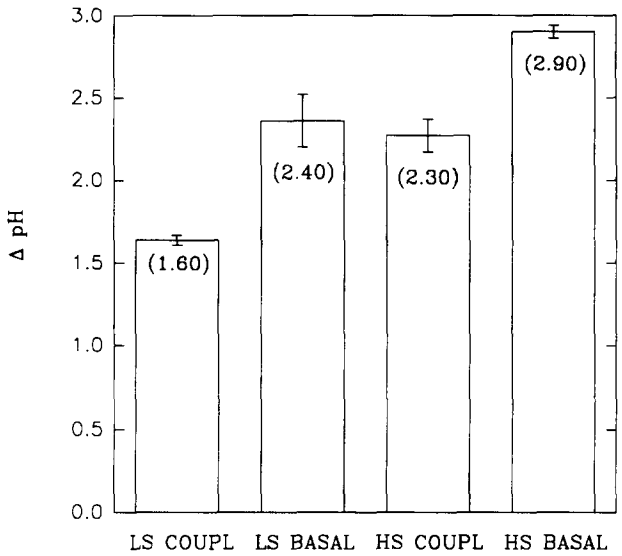

Fig. 6. ΔpH determination in low-salt and high-salt-stored thylakoids in basal and coupled conditions. The conditions were as described in the methods section. The lumen pH values were calculated by determining the ratio of the respective $\Delta F_{450}/\Delta F_{405}$ and extrapolating these ratio to the corresponding pH values using the HPTS-loaded thylakoid pH calibration (Fig. 3) Numbers in brackets are ΔpH values. The light intensity was 24 μE $m^{-2}s^{-1}$. The data are the average of twenty separate measurements using five different thylakoid preparations.

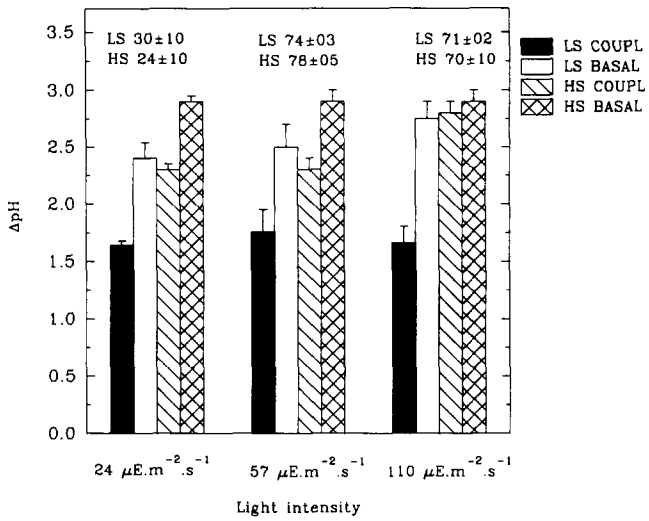

Fig. 7. ΔpH determination in low-salt and high-salt-stored thylakoids in basal and coupled conditions at three different actinic light intensities. Assay conditions were similar to Fig. 6 except the actinic light intensity was increased from 24 to 57 and 110 μE $m^{-2}s^{-1}$. The numbers above the bar graphs are the rates of ATP formation determined in separate assays of the same thylakoid preparations used for the ΔpH determination. The ATP formation was done using the luciferin-luciferase method in a steady state mode as reported by Renganathan et al. [13]. The reaction conditions were the same as for the ΔpH determination with the addition of 5 μM diadenosine pentaphosphate (to inhibit adenylate kinase activity) and luciferin-luciferase reagents (cf. Ref. 13) and omitting the hexokinase.

the ATP formation rates in Fig. 7 and Table II are rather low, but that is in part owing to the external pH being 8.9, and it is known that the pH curve for ATP formation drops sharply between pH 8 and 8.9; e.g., in Ref. 41, from near 290 to near 40 μmol ATP (mg Chl h)$^{-1}$. However, as shown in Table II, the washing steps used to remove the external HPTS after dye loading also contribute to lower phosphorylation activities.

ΔpH versus external pH

The high external pH (8.9) used for the ΔpH measurements, shown in Figs. 6 and 7, permitted making accurate determinations even when the internal pH dropped to near 6 (in the high-salt, basal sample (Fig. 6)). Because the pH calibration curve becomes quite flat below pH 6.0 (Fig. 3 and 5), we cannot extend the measurements into the range of pH 5.5. Nonetheless, for the coupled condition at an external pH of 8.9 with low-salt-stored thylakoids, the ΔpH values were near 1.5–1.6 (Figs. 6 and 7; i.e., the lumen pH was near 7.3, in the range of good pH sensitivity), and it seemed likely that with the low-salt-stored, coupled case the ΔpH could be measured at lower external pH with the measurement staying in the usable part of the calibration curve. This was the case, as seen in Fig. 8, where the lumen pH under coupled conditions with low-salt

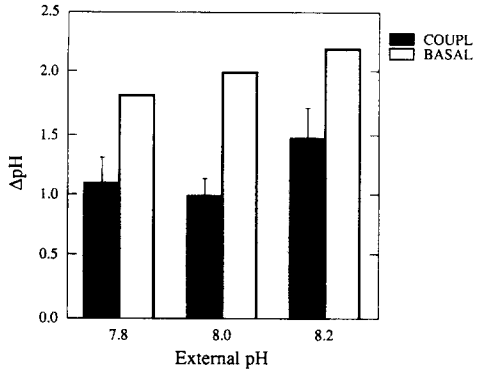

Fig. 8. ΔpH determined with low-salt-stored thylakoids at pH 7.8, 8.0 and 8.2 under coupled and basal conditions. The assay conditions were similar to those of Fig. 6, except that Tricine buffer was used in place of the Taps buffer used at pH 8.9.

stored thylakoids reached no lower than about 6.7 (for the pH 7.8 external condition) and near 7.0 for the pH 8.0 and 8.2 (external) experiments; in all cases well within the sensitivity range for the HPTS calibration. For basal conditions, however, the lumen pH reached close to pH 6 in all three cases, and the calibration curve is so flat there that it is not possible to specify the lumen pH better than to say that it reached a pH $\leq$ 6.0 in the basal conditions. When hexokinase was omitted for the coupled conditions, experiments at external pH values near 8, there was no detectable difference in the measured lumenal pH from the case with hexokinase and glucose present. Typical data for pH 8.2 external (without hexokinase, glucose) were $\Delta pH = 1.0 \pm 0.1$ for low-salt-stored thylakoids, essentially identical to samples assayed with hexokinase, glucose present.

Using the pH 8.2 assay medium, an experiment was done wherein the HPTS was loaded with the normal 200 mM sorbitol-containing medium but the ΔpH assay was done in media having no sorbitol to test for the effect of swelling the loaded thylakoids (decreasing the lumenal concentration of HPTS). The ΔpH in the low osmotic pressure medium under coupled conditions was 1.19 ± 0.01, a value similar within experimental error to the value obtained when the ΔpH assay was done in the usual way with 200 mM sorbitol present (Table 3). In the same experiment, HPTS-loading was done with zero sorbitol and the ΔpH assay was also in medium having no sorbitol, and the ΔpH assay gave a value of 1.11 ± 0.13. Under basal conditions the ΔpH was > 2.2 for all these experiments, but as mentioned above, the pH response of the dye becomes too small to use at pH values near or below pH 6.0. Those results support the point discussed above (cf. Fig. 5) that the ratio method is not influenced by the dye concentration in the space being assayed. The samples loaded in 200 mM sorbitol are expected to have a much smaller lumenal volume than those loaded in media without sorbitol, and consequently less HPTS. Transferring samples loaded in 200 mM sorbitol to the assay medium without sorbitol would dilute the lumenal HPTS considerably, yet there was no significant change in the fluorescence ratio. Although sorbitol is more permeable across thylakoid membranes than sucrose, having a $t_{1/2}$ of entry at 4°C of 70 min compared to that of sucrose ($t_{1/2} > 180$ min) [37], in a shift from 200 mM sorbitol (loading, 30 min) to the zero sorbitol in the assay medium (line 3, Table III) there would be a marked swelling. Hence, the assay results on line 3, Table III, being in the same range of calculated ΔpH as the assays done in 200 mM sorbitol, support the notion that changes in dye concentration resulting from such a short-term swelling (the assays were done within 3 min after transfer from the 200 mM sorbitol to the zero sorbitol) report similar fluorescence ratios, and therefore similar ΔpH values as in the less swollen sample.

Effect of HPTS in the external medium

It seems quite clear that the light-dependent HPTS fluorescence signal, as we have described it above, is a response to changes in lumen pH. However, additional controls are useful to check for other factors which may influence the fluorescence signal. One consideration is whether external HPTS could contribute to fluorescence changes, owing perhaps to changes in dye-external membrane surface interactions in response to energization-dependent membrane conformation changes (even though the suspending medium was heavily buffered (20 mM Tricine or Taps, for example)). To test this, HPTS was added to the thylakoid suspension having no HPTS loaded into the lumen, to test whether external HPTS gives any fluorescence change upon energization of the membranes.

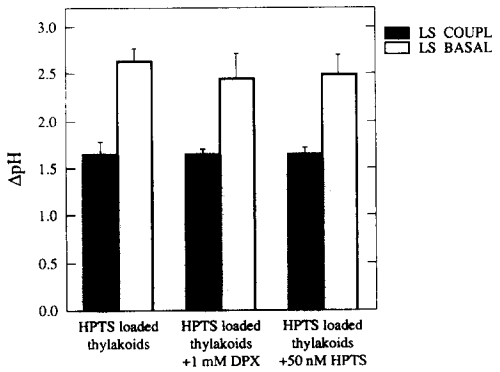

Fig. 9. Effect of varying the external (free) dye concentration on ΔpH determination in HPTS loaded low-salt-stored thylakoids. Assay conditions were the same as in Fig. 6, except where required 1 mM DPX or 50 nM HPTS was added to decrease or increase the external free dye concentration. 1 mM DPX addition decreased the fluorescence to 0.6 and 50 nM HPTS increased the fluorescence to 2-fold the value of HPTS loaded thylakoid fluorescence. ΔpH values were determined from the average of three experiments.

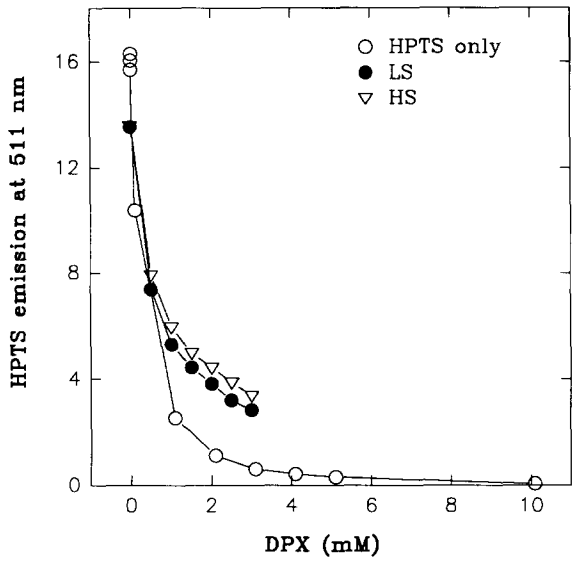

Fig. 10. Quenching of HPTS fluorescence by *p*-xylenebispyridium bromide. (DPX) Low-salt-stored, •, or high-salt-stored, ▽, thylakoids were loaded with HPTS as in Fig. 6 and assayed in pH 8.9 assay media with the indicated concentration of DPX added to the assay cuvette. The fluorescence signal was monitored at 511 nm with excitation at 450 nm. ○, HPTS only, indicates that 10 μM HPTS, without thylakoids, was present in the assay medium.

With 50 nM HPTS added (giving about the same total fluorescence signal as normally obtained with HPTS-loaded thylakoids), there was no detectable fluorescence change after switching on and off the actinic light (data not shown). A different approach, that of adding 50 nM HPTS to a reaction with HPTS-loaded thylakoids just before giving the light cycle to initiate the ΔpH signal, is shown in Fig. 9. The 50 nM external HPTS, while contributing significantly to the total fluorescence signal (8000 counts minus externally added HPTS and 16000 counts with the added 50 nM HPTS), did not alter the calculated ΔpH (light-dependent signal). Those results are consistent with the notion that the fluorescence changes observed originate from internal HPTS. Thus, it is concluded that external dye does not contribute to the light-dependent HPTS fluorescence changes we observe during energization.

Another approach was to add a quencher of HPTS fluorescence such as *p*-xylenebispyridium bromide (DPX) (cf. Ref. 38 and literature from Molecular Probes, Eugene, OR). Concentration curves for DPX quenching of HPTS fluorescence in buffer with no thylakoids showed 95% quenching at 4 mM DPX and 82% quenching at 1 mM (Fig. 10, open circles). Thylakoids loaded with HPTS in the usual way also showed significant but less fluorescence quenching. Approximately 80% of the 511 nm fluorescence was quenched in such samples at 3 mM DPX. That level of quenching HPTS fluorescence in suspensions of dye-loaded thylakoids seemed too high to be explained only by quenching external dye, rather we suspected that DPX might be entering the thylakoid lumen. We tested this by measuring the DPX effect on the time dependence of the light-induced fluorescence changes, reasoning that the entry of DPX into the lumen would quench some of the fluorescence in a time-dependent way. However, given the principle that the *ratio* of the ΔF changes is independent of lumenal dye concentration, the ΔpH calculated from the ΔF ratio should stay constant in the face of a decrease in 511 nm fluores-

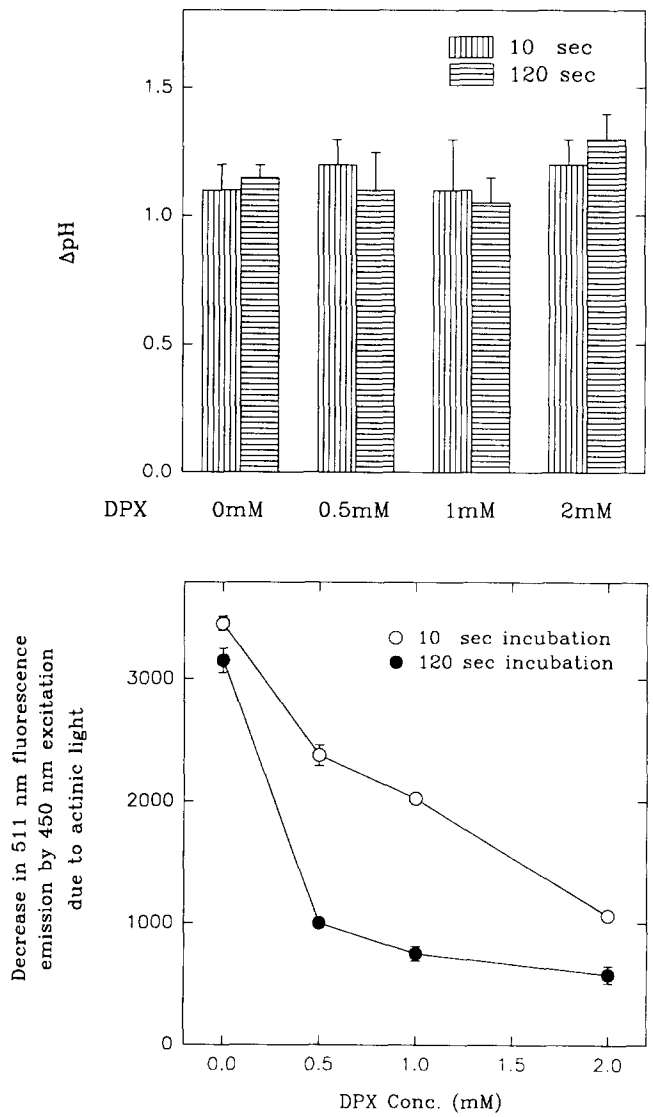

Fig. 11. Effect of 10 s or 2 min incubation in the fluorescence quencher, DPX, on the calculated ΔpH (top) and on the 511 nm fluorescence excited by 450 nm light (bottom) *Top.* Low-salt-stored thylakoids were loaded with HPTS and assayed as described in Fig. 8 at an external pH of 8.2. The indicated DPX concentrations were added to the assay cuvette and light-dependent fluorescence was followed starting at 10 s after DPX addition or after 2 min in the presence of DPX. *Bottom.* The 450 nm excitation wavelength signal plotted versus DPX concentration at the two time points indicated. The greater drop in the ΔF signal with DPX at 2 min is consistent with DPX penetrating the lumen significantly more after 2 min compared to penetration after 10 s exposure.

cence elicited by either 405 or 450 nm excitation. Fig. 11 shows such an experiment, including the light-dependent ΔF from 450 nm excitation (bottom panel) and the ΔpH calculated from the ΔF ratio (top panel) as a function of time that DPX was present in the thylakoid suspension. It is clear that between zero and 2 mM DPX the calculated ΔpH was not influenced by the quencher (top panel, Fig. 11). However, there was significantly more quenching of the 450 nm excitation of fluorescence (the 405 nm excitation was similarly quenched, data not shown) at 2 min compared to 10 s exposure to DPX (bottom panel). Thus, it seems likely that DPX does penetrate thylakoids. The data of Fig. 9 (center compared to left side data) indicate that the 1 mM DPX had no influence on the estimated light-dependent ΔpH for either basal or coupled conditions.

	○	●	▽
Y-intercept	2.56	11	26
slope	87	526	2223
r	0.90	0.90	0.90
X-intercept	−0.029	−0.021	−0.012
Dissociation constant	34 μM	48 μM	85 μM

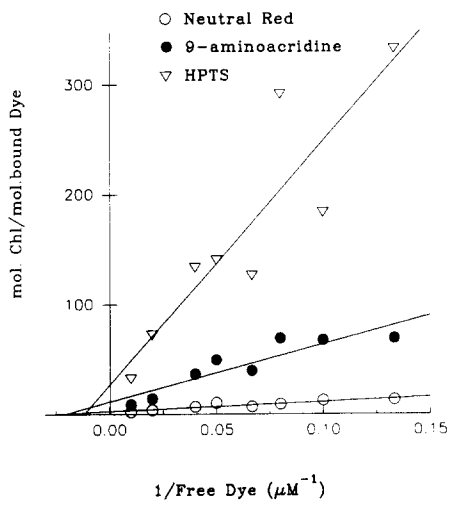

Fig. 12. Neutral red, 9-aminoacridine and HPTS binding to thylakoid membranes. 5–100 μM of dye concentrations were used to determine their binding constant to thylakoid membranes. Inverted value of the x-axis intercept indicates the binding constant of these dyes. Various (5 to 100 μM) concentrations of the dyes were added to ΔpH assay buffer which consisted of 100 μg Chl/ml thylakoids, 200 mM sorbitol, 20 mM Taps-KOH (pH 8.9), 3 mM $MgCl_2$, 20 mM KCl, 100 nm nonactin and incubated for 30 s. After incubation, the suspensions were centrifuged in a microcentrifuge for 10 min at 10°C and the supernatant was taken for the estimation of dye concentrations. The concentration of neutral red, 9-aminoacridine and HPTS was measured spectrophotometrically at 524, 420 and 450 nm. To correct for the residual chlorophyll concentration in the supernatant, supernatant from a thylakoid suspension with no dye was measured at 524, 420 and 450 nm and subtracted from the respective wavelengths of the dye binding measurements. The amount of bound dye was estimated by subtracting the free dye from the total dye concentration.

HPTS–thylakoid interaction

It is useful to compare the binding affinity of HPTS to the membrane with that of other pH-indicating dyes used for determining pH relationships in thylakoids, namely neutral red (NR) and 9-aminoacridine (9-AA). Fig. 12 shows a double-reciprocal plot of the binding of each of the three dyes to thylakoids. As expected, the negatively charged HPTS has a much lower affinity constant (or higher K_m) for binding than either NR or 9-AA. The K_m values determined from the data of Fig. 12 were HPTS, 86 μM; 9-AA, 48 μM; and NR, 34 μM.

Discussion

These results provide additional evidence that thylakoids can form either localized or delocalized energy coupling proton gradients. We proposed this energy coupling model in earlier work [7,13,34], based on a wide array of experiments, including one experiment wherein ΔpH was measured by the [^{14}C]methylamine distribution method but only for coupled conditions using low-salt- and high-salt-stored thylakoids [13]. In those experiments, the calculated ΔpH (pH 8.0 external) for low-salt-stored thylakoids at a phosphorylation rate of 100 μmol ATP (mg Chl h)$^{-1}$ was near ΔpH = 1.5 and for high-salt-stored membranes ΔpH = 2.0. However, the ΔpH values were not measured under other conditions of interest, and a technique easier to use than the amine distribution has a great value for this and other studies. This work presents a convenient, rapid method for determining lumenal pH... a worthy end in itself and especially useful for testing predictions of the dual energy coupling gradient hypothesis we made from our earlier, less direct methods. In this work we used the advantages of the fluorescent dye method in assessing the lumen pH – via the pH dependence of lumenally-located HPTS fluorescence – during various situations of basal or coupled electron (H^+) flow in the two types of thylakoid suggested to be capable of localized $\Delta\tilde{\mu}_{H^+}$ coupling (low-salt-stored) or delocalized coupling (high-salt-stored).

That the HPTS fluorescence ratio signal accurately measures the lumen pH is supported by the close correspondence of the pH calibration curves of the fluorescence ratio $\Delta F_{450}/\Delta F_{405}$ from pH 8.9 to 6.0, for the dye without thylakoids and with dye-loaded thylakoids (Figs. 1–3). The ratio method is especially useful in that the pH dependence of the calibration curve is *independent of dye concentration in the interior space being assayed*, a point already established for similar dyes such as Fura-2 used in a variety of cells by

Tsien and colleagues [26,32]. In the thylakoid preparations used here, the ratio signal was independent of HPTS concentration in the lumen, as indicated by the data of Fig. 5 (where different dye concentrations were used in loading) and Table III (where the thylakoids were osmotically swollen in the assay medium). In both cases one logically predicts that the lumenal dye concentration was significantly different between the treatments, but the light-dependent ratio signal was similar and computed out as a similar ΔpH. This point is also shown in the data of Fig. 11, where the quencher DPX appeared to penetrate the lumen to a significant extent in 120 s so as to partially quench the light-dependent fluorescence transients at 450 nm (shown in Fig. 11) and 405 nm (not shown) excitation, but the calculated ΔpH derived from the *ratio* of ΔF did not change even though the component wavelength contributions to ΔF did change (were quenched). Those fluorescence changes, indicative of acidification, are logically owing to HPTS in the lumen, and the DPX apparently reaches the lumen and quenches part of the HPTS without disturbing the calculated ΔpH value (Fig. 11, top). If the ratio pH calibration curve is dye concentration-independent, then it follows that the ratio should not be significantly altered by changes in lumen volume which may occur in light/dark transients or other treatments which may change the lumen volume or the dye loading.

Control experiments established that the dye-loading and washing steps, while decreasing phosphorylating activity, did not cause the loss of the localized coupling response in the low-salt-stored thylakoids. This was assayed for in two ways; i.e., by observing the lack of effect of a permeable amine (HEM) on the ATP formation onset lag [6,7] in single-turnover flash trains (Table I) and by measuring the transient delay in steady illumination-driven ATP formation [13] induced by addition of a permeable amine after 24 s of steady light (Table II). As expected from previous experience with centrifuging and resuspending thylakoids, the washing steps clearly decreased the efficiency of the ATP formation in 5 Hz flash trains (Table I, measured as ATP yield per flash) and the steady-light driven ATP formation rate (Table II). However, the low-salt-stored thylakoids maintained the characteristics we attribute to localized energy coupling, whereas the high-salt-stored thylakoids responded as in the earlier work, with the characteristics of a delocalized system [6,7,13].

There always is concern that a fluorescence probe signal, here attributed to pH changes in the lumen volume, could have a contribution from membrane-dye interactions in addition to, or instead of, the presumed lumenal pH change. Such spurious signals do not seem to occur in this case based on the following evidence or arguments: (a) no ΔF signal occurred when 5 μM nigericin and K^+ kept the H^+ gradient from developing (Fig. 4, bottom). With nigericin plus K^+, electron transport is fast and the initial pulse of $\Delta\Psi$ occurs as in the control and similarly rapidly relaxes to the low (≈ 10 mV) steady illumination value characteristic of thylakoids. Hence, if the ΔF signal seen with HPTS were caused by membrane potential effects, it would have been observed in the presence of nigericin, but that was not the case. (b) Externally located HPTS with thylakoids not loaded with the dye gave no light-dependent ΔF, nor did added external HPTS to dye-loaded thylakoids change the ΔF ratio (compared to a dye-loaded sample with no externally added dye) observed in light-dark cycles (Fig. 9).

When this system was used at an external pH of 8.9 for estimating ΔpH values across the thylakoid during steady illumination, the data of Fig. 6 show that thylakoids operating in the localized coupling mode (predicted from other criteria) did indeed have a ΔpH too low to account for energizing ATP formation if a lumen-to-outside $\Delta\tilde{\mu}_{H^+}$ gradient were driving the CF_0-CF_1 energy coupling. The ΔpH needed to match the calculated ΔG_{ATP} (from $\Delta G_{ATP} \geq n\Delta\tilde{\mu}_{H^+}$ where n is the stoichiometric H^+ coupling coefficient), see footnote *, is 2.51 ΔpH without hexokinase/glucose and 1.97 ΔpH with the ATP trap present. The ΔpH calculated from the HPTS data was only 1.60 ± 0.03 for the external pH 8.9 conditions (Fig. 6).

For the external pH 8 coupled conditions, the ΔG_{ATP} would require a ΔpH near 2.2 units with no hexokinase and 1.64 units with hexokinase/glucose present. The observed ΔpH for the low-salt-stored thylakoids estimated with the HPTS method gave a value of ΔpH = 1.0 ± 0.1 (Fig. 8), far too low to meet the energy demands if bulk phase-to-bulk phase ΔpH were the driving force for ATP formation.

The high-salt-stored thylakoids, predicted by the earlier criteria to be in a delocalized mode, had a ΔpH near 2.3–2.6 under coupling conditions at low light intensity (24 $\mu E\ m^{-2}\ s^{-1}$, Fig. 6 and 7) and near 2.8 at higher light intensity (110 $\mu E\ m^{-2}\ s^{-1}$, Fig. 7). Those thylakoids were clearly developing a sufficient ΔpH to meet the energetic demands predicted for bulk-to-bulk energy coupling. It is expected that the ΔpH (lumen-to-external) would increase with increasing light intensity for delocalized coupling as the rate of electron and H^+ transport exceeds the capacity of the CF_1 phosphorylation rate to keep up, and that is what appears to be occurring between 57 and 110 $\mu E\ m^{-2}\ s^{-1}$ (Fig. 7). The maximum rate of ATP formation, owing to the effects of the washing steps (see above), the pH 8.9 assay conditions and to the 10°C temperature used was rather low, about 70 μmol ATP(mg Chl h)$^{-1}$.

The low-salt-stored thylakoids under coupling conditions maintained a lumen-to-external ΔpH near 1.6 over the entire intensity range (Fig. 7), consistent with the critical protonmotive force driving the ATP forma-

tion being localized in those thylakoids. Yet, the same low-salt-stored thylakoids, when energized by basal electron/H^+ flow (no ADP present), produced a lumen-to-external ΔpH near 2.4 in low intensity and near 2.8 in high light (Fig. 7). This indicates that there are no unusual constraints placed on the low-salt-stored thylakoids which predispose them to, for example, quench the HPTS fluorescence signal more than with the high-salt-stored sample.

When the external pH was dropped to 7.8–8.2 with low-salt stored thylakoids (Fig. 8), the HPTS fluorescence technique reported a ΔpH of near 1.0 units for pH 7.8 and 8.0 and 1.3 units at pH 8.2 under coupled conditions. Thus, the internal phase reached a pH near 7.0, still easily within the sensitivity of the calibration. The low-salt-stored thylakoids under basal conditions showed the internal pH dropping to near (or less than) pH 6.0 (Fig. 8), too acidic to accurately determine, but clearly significantly more acidic than for the coupled case. The energetic threshold for ATP formation calculated for pH 8.0 (see footnote) predicts a ΔpH of 1.64 is needed when hexokinase/glucose was present and 2.18 without the ATP trap. In order for the bulk phase protonmotive force to provide the necessary energy (at 3 H^+/ATP), a $\Delta\Psi$ of 39 mV would have to have been present in addition to the 1.0 unit ΔpH to supply the necessary energy (Δp = 2.18 kcal × 1 mV/0.023 kcal = 95 mV; Δp = 95 mV = 56.2 mV (for the observed ΔpH of 1) + $\Delta\Psi$, therefore the necessary $\Delta\Psi$ would have to be 39 mV).

In these experiments we used a $\Delta\Psi$-collapsing ionophore (nonactin with total K^+ near 30 mM) which suppresses $\Delta\Psi$ build-up. Moreover, it has been shown that in steady illumination, even with no ionophores present, the $\Delta\Psi$ across the thylakoid membrane is near or less than 10 mV [16,39]. Therefore, we can exclude the $\Delta\Psi$ as making up the energy shortfall noted for bulk-to-bulk ΔP in the low-salt-stored thylakoid case. In the earlier single-turnover flash studies we measured the 515 nm electrochromic shift as a membrane potential indicator for low- and high-salt-stored thylakoids and found virtually identical patterns for the rise and the decay phases [7,13,34,40]. Therefore, we cannot expect the low-salt-stored samples to have a different (greater) $\Delta\Psi$ component than the high-salt-stored case. It should be kept in mind that the assays we have used for phosphorylation, $\Delta\Psi$ measurements and the HPTS ΔF are all done in an identical medium, only the storage was in low- or high-salt. In spite of our having extensively studied, discussed, and clearly ruled out [7,13,34,40] a possible $\Delta\Psi$ component being greater in the low-salt compared to high-salt-stored thylakoids (thus making those thylakoids not show the ATP formation onset lag increase owing to a permeable buffer), the question remains in the minds of some colleagues (and one reviewer of this manuscript). To make clear once again, by the accepted criteria for measuring the rise and decay of the 515 nm electrochromic shift, there were no differences in the $\Delta\Psi$ indicator between low- or high-salt-*stored* thylakoids (but assayed in an identical medium) either in the basal (−ADP) mode (Fig. 7 of Ref. 7) or the coupled mode (Fig. 7 of Ref. 13).

Following the above arguments, these data support the hypothesis [6,7,34] that low-salt-stored thylakoids can maintain a localized energy coupling protonmotive force. However, we retain the notion that a $\Delta\tilde{\mu}_{H^+}$ gradient is the driving force for ATP formation, and therefore it is concluded that in the thylakoid sequestered domains the ΔpH drop between the domains and the external phase must exceed the energetic threshold ΔpH = 2.18 unit value calculated to be necessary for pH 8.0 or be > 2.51 units for the pH 8.9 conditions.

* From the calculated ΔG_{ATP} and assuming $n = 3$ H^+ per ATP as the stoichiometric coupling coefficient, and assuming that the electric field contribution to the protonmotive force is negligible, we can calculate the ΔpH necessary to overcome the thermodynamic energy requirement to initiate ATP formation. The ΔG_{ATP} for pH 8.9 and 10°C was calculated as 7.63 kcal/mol using the $\Delta G°$ = 8.93 kcal/mol of Rosing and Slater [35] (extrapolated to 10°C) and 0.2 mM ADP, 5 mM P_i, 3 mM $MgCl_2$ and 10^{-7} M ATP. In the absence of the hexokinase/glucose trap we measured the ATP level in the reaction medium to be 4 μM (using the luciferase method) This gives a ΔG_{ATP} of 9.71 kcal/mol compared to the 7.63 kcal/mol for the [ATP] = 10^{-7} M case. The low ATP concentration in the presence of hexokinase/glucose is an estimate arrived at as follows: with hexokinase and glucose in the coupled ΔpH assay medium (with 0.2 mM ADP) no net ATP formation was observed in steady illumination, and the sensitivity of our assay showed that the ATP concentration was < 0.2 μM ATP during continuous illumination. In the absence of the hexokinase, the ATP formation rate was measured as 66 μmol ATP (h mg Chl)$^{-1}$. Moreover, by adding an aliquot of standard ATP to a similar reaction medium having the hexokinase/glucose trap supplemented with luciferin-luciferase (see Methods), the luminescence signal gave a sharp rise upon adding the ATP but the signal dropped quickly (< 1 s) back to the baseline. Even after adding 5 × ATP (0.89 μM final), the signal peaked and dropped to the baseline in about 1 sec. Therefore, we conclude that the hexokinase/glucose trap maintained the ATP level near 0.1 μM. However, this is something of a moot point since the HPTS ΔF signals gave virtually identical calculated ΔpH values with or without the ATP trap.

The calculated ΔpH needed to reach the threshold energization (from $\Delta G_{ATP} = n\Delta\tilde{\mu}_{H^+}$) is 1.97 ΔpH for pH 8.9 conditions with hexokinase/glucose present and 2.51 ΔpH without the ATP trap. For pH 8.0 we used a $\Delta G°_{ATP}$ = 7.66 kcal/mol, giving a ΔG_{ATP} = 6.36 kcal/mol with hexokinase/glucose, resulting in a calculated ΔpH of 1.64 for the energetic threshold. With no hexokinase/glucose, the values for pH 8.0 are ΔG_{ATP} = 8.44 kcal/mol and a required threshold ΔpH of 2.27. The observed ΔpH values for coupled conditions in low-salt-stored membranes were the same with or without the hexokinase/glucose trap, and in all external pH conditions used the observed ΔpH was significantly less than the energetic requirement predicted for bulk-to-bulk phase coupling.

Obviously, under the "localized" energy coupling conditions, there was a certain amount of lumenal acidification, albeit not sufficient to energize ATP formation by the transmembrane ΔpH nor sufficient to open the putative Ca^{2+}-regulated H^+ flux gate we have postulated as operating at the lumenal side of the CF_0 H^+ channel [12,34,36]. The origin of the spillover of H^+ ions into the lumen under these conditions is not clear... it could be caused by a fraction of the redox chains being disconnected from the proposed sequestered domains, or a certain leakiness probability associated with the domains. The resolution of this question remains for future studies.

Various control experiments showed that the HPTS pH probe is a robust indicator of lumen pH. In particular, any HPTS leaking into the external medium does not influence the calculated internal pH (Figs. 9 and 10). As discussed in the Results section and in the Appendix, the advantage of using the ratio of wavelengths method to compute the pH dependence of the fluorescence signal is that the internal pH calibration is independent of dye concentration inside and internal volume (cf. Fig. 3).

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Concluding remarks

The results of these lumen pH measurements under the predicted localized or delocalized $\Delta\tilde{\mu}_{H^+}$ gradients add additional, strong, evidence to an already strong case supporting the hypothesis that thylakoids can maintain a membrane-localized energization under some conditions or express a transmembrane energization mode under other conditions [6,7,34]. We have previously suggested that the reversible gating of the energy coupling proton gradient between membrane-localized domains or a delocalized energization is controlled by Ca^{2+} ions bound at the lumenal side of the CF_0 H^+ channel [12,34,36].

References

1 Ort, D.R., Dilley, R.A. and Good, N.E. (1976) Biochim. Biophys. Acta 449, 108–124
2 Graan, T., Flores, S. and Ort, D.R. (1981) In Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), Developments in Biochemistry, Vol 20, 25–34, Elsevier/North-Holland, Amsterdam.
3 Horner, R.D. and Moudrianakis, E.N. (1983) J. Biol. Chem. 258, 11643–11647.
4 Pick, U., Weiss, M. and Rottenberg, H. (1987) Biochemistry 26, 8295–8302.
5 Sigalet, C., Haraux, F., De Kouchkovsky, F., Hung, S. and De Kouchkovsky, Y. (1985) Biochim. Biophys. Acta 809, 403–413.
6 Beard, W.A. and Dilley, R.A. (1986) FEBS Lett. 201, 57–62.
7 Beard, W.A. and Dilley, R.A. (1988) J. Bioenerg. Biomembr. 20, 129–154.
8 Davenport, J.W. and McCarty, R.E. (1980) Biochim. Biophys. Acta 589, 353–357.
9 Vinkler, C., Avron, M. and Boyer, P.D. (1980) J. Biol. Chem. 255, 2263–2266.
10 Gräber, P. (1982) Current Topics Membr. Trans. 16, 215–245.
11 Junge, W. (1987) Proc. Natl. Acad. Sci. USA 84, 7084–7088.
12 Chiang, G. and Dilley, R.A. (1987) Biochemistry 26, 4911–4916.
13 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.
14 Beard, W.A., Chiang, G.G., and Dilley, R.A. (1988) J. Bioenerg. Biomemb. 20, 107–128.
15 Gaensslen, R.E. and McCarty, R.E. (1971) Arch. Biochem. Biophys. 147, 55–65.
16 Rottenberg, H., Grunwald, T. and Avron, M. 1972) Eur. J. Biochem. 25, 54–63.
17 Allnutt, F.C.T., Atta-Asafo-Adjei, E. and Dilley, R.A. (1989) J. Bioenerg. Biomembr. 21, 535–551.
18 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.
19 DeBenedetti and Garlaschi, F.or. (1977) J. Bioenerg. Biomemb. 9, 195–201.
20 Hope, A.B. and Matthews, D.B. (1985) Austral. J. Plant Physiol. 12, 9–19.
21 Bizouarn, T., De Kouchkovsky, Y. and Haraux, F. (1989) Biochim. Biophys. Acta 974, 104–113.
22 Junge, W., Ausländer, W., McGeer, A.J. and Runge, T. (1979) Biochim. Biophys. Acta 546, 121–141.
23 De Wolf, F.A., Groen, B.H., Van Houte, L.P.A., Peters, F.A.L.J., Krab, K. and Kraayenhof, R. (1985) Biochim, Biophys. Acta 809, 204–214.
24 Junge, W., Schönnecht, G. and Förster, V. (1986) Biochim. Biophys. Acta 852, 93–99.
25 Blumenfeld, L.A., Davydov, R.M. and Tikhonov, A.N. (1989) J. Mol. Liquids 42, 231–253.
26 Tsien, R.Y. and Poenie, M. (1986) Trends Biochem. Sci. 11, 450–455.
27 Rink, T.J., Tsien, R.Y. and Pozzan, T. (1982) J. Cell. Biol. 95, 189–196.
28 Wolfbeis, O.S., Furlinger, E., Kroneis, H. and Marsoner, N. (1983) Fresenius Z. Anal. Chem. 314, 119–124.
29 Arnon, D.I. (1949) Plant Physiol. 27–44.
30 Bush, D.S. and Jones, R.L. (1988) Cell Calcium 8, 455–472.
31 Bush, D.S. and Jones, R.L. (1988) Eur. J. Cell Biol 46, 466–469.
32 Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3448.
33 Heldt, H.W., Werdan, K., Milovancev, M. and Geller, G. (1973) Biochim. Biophys. Acta 314, 224–241.
34 Dilley, R.A. (1991) Curr. Topics Bioenerg. 16, 265–318.
35 Rosing, J. and Slater, E.C. (1972) Biochim. Biophys. Acta 267, 275–290.
36 Chiang, G.G., Wooten, D.C. and Dilley, R.A. (1992) Biochemistry 31, 5808–5819.
37 Flores, S., Graan, T. and Ort, D.R. (1983) Photobiochem. Photobiophys. 6, 293–304.
38 Smolarsky, M., Teitelbaum, D., Sela, M. and Gitler, C. (1977) J. Immunol. Methods 15, 255–265.
39 Vredenberg, W.J. (1974) Proceedings of the 3rd International Congress on Photosynthesis (Avron, M., ed.), Vol. II, 929–939, Elsevier, Amsterdam.

40 Theg, S.M., Chiang, G. and Dilley, R.A. (1988) J. Biol. Chem. 263, 673–681.
41 Wright, G.D., Izawa, S. and Good, N.E. (1965) Biochem. Biophys. Res. Commun. 21, 438–442.

Appendix

Assumption: there is some amount of dye leakage from the lumen into the assay buffer during the sample preparation, the time for which may be about 5 min.

Let F_d = fluorescence seen immediately before the onset of actinic illumination

F_l = steady state fluorescence level during actinic illumination

D_{id} = dye inside the lumen before the onset of actinic illumination

D_{od} = dye outside (in the assay buffer) before the onset of actinic illumination

D_{il} = dye inside the lumen during actinic illumination

D_{ol} = dye outside (in the assay buffer) the lumen during actinic illumination

Fluorescence signal at F_d

$$F_{d450} = D_{id450} + D_{od450}$$
$$F_{d405} = D_{id405} + D_{od405}$$

Fluorescence signal at F_l

$$F_{l450} = D_{il450} + D_{ol450}$$
$$F_{l405} = D_{il405} + D_{ol405}$$

then the $F450/F405$ ratio of the lumenal dye between F_d and F_l

$$= \frac{F_{d450} - F_{l450}}{F_{d405} - F_{l405}}$$

$$\frac{(D_{id450} + D_{od450}) - (D_{il450} + D_{ol450})}{(D_{id405} + D_{od405}) - (D_{il405} + D_{ol405})}$$

By giving a short 10 s actinic illumination and assuming that there is no significant dye leakage during this period

$$D_{od450} = D_{ol450}$$

and

$$D_{od405} = D_{ol405}$$

then

$$F450/F405 = \frac{D_{id450} - D_{il450}}{D_{id405} - D_{il405}}$$

then assuming all the dye inside the lumen senses acidification and fluoresces

$$D_{id450} - D_{il450} = \Delta F_{450}$$

$$D_{id405} - D_{il405} = \Delta F_{405}$$

$$F450/F405 = \Delta F_{450}/\Delta F_{405}$$