Protons in the Thylakoid Membrane-sequestered Domains Can Directly Pass Through the Coupling Factor during ATP Synthesis in Flashing Light*

(Received for publication, February 24, 1987)

Steven M. Theg‡, Gisela Chiang, and Richard A. Dilley§

From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Thylakoid membranes contain sequestered domains in which protons are held in a metastable state out of equilibrium with those in the inner (lumen) or the outer aqueous bulk phases unless the membranes are made leaky by, for instance, the addition of uncouplers. Previously, it has not been clear whether such sequestered domain protons are: 1) directly on a localized pathway into the CF₀-CF₁; 2) on an ultimately delocalized pathway comprising domains → lumen → CF₀-CF₁; or 3) perhaps not importantly involved in any way with proton gradient-linked ATP formation. Recent developments now permit a test of the above possibilities.

The test for the possible mode of involvement of domain protons in energy coupling utilized single turnover flashes to energize electric field-driven ATP formation, as influenced by both the proton depletion level of the sequestered domains and whether the thylakoids were prepared in a way to show either a localized or delocalized proton gradient response in ATP formation (this response is reversibly controlled by incubation in low (localized coupling mode) or high KCl (delocalized mode) incubations (Beard, W. A., and Dilley, R. A. (1986) FEBS Lett. 201, 57–62; Chiang, G. and Dilley, R. A. (1987) Biochemistry 26, 4911–4916)).

Using thylakoids showing delocalized coupling responses, there was no influence of the reversible depletion of the sequestered domain buffering pool on the Δψ-driven ATP formation onset lag. Luminal protons rather than domain protons in that case appear to be the first to be driven through the CF₀-CF₁ complex by the Δψ field. However, using thylakoids prepared to be in the localized gradient coupling mode, protons in the domains, rather than those in the lumen, are the first to pass through the CF₀-CF₁ complex in the onset of energization. We conclude that the sequestered domain protons, in the latter case only, are obligatorily in the main proton diffusion pathway for energization of ATP formation and are the first protons (rather than lumen protons) driven through the CF₁-CF₀ complex by a Δψ field.

Several laboratories have reported evidence consistent with the occurrence of proton-buffering groups contained within a sequestered domain of chloroplast thylakoid membranes (1–11). Lysine amine groups with anomalously low pKₐ values (7.1–7.8) are the only groups thus far identified (1, 3, 7), but other groups with lower pKₐ values (probably carbamoyls) have been implicated by recent results (12). About 30–40 nmol (mg Chl)⁻¹ of the pKₐ = 7.5 lysine groups occur in the sequestered domains, the quantitative estimate being derived from direct pH measurements of H⁺ efflux in the dark upon addition of uncoupler (1, 6, 11) and the closely corresponding uncoupler-induced extra acetylation of amino groups with [³H]acetic anhydride (1, 11).

A critical question is whether the sequestered domain protons are "in transit" on a pathway to the CF₀-CF₁ complex or whether they are on a side pathway unrelated to proton flux to the coupling complex. In the presence of valinomycin and K⁺, which blocks the Δψ contribution to the proton-motive force (Δp), Dilley and Schreiber (10) showed that the number of flashes required to build a ΔpH sufficient to overcome the thermodynamic threshold for ATP synthesis (the "onset flash lag") was related to the protonation state of the domains. That is, about 10–12 more flashes were required to reach the energization threshold if the domains were emptied of the metastable protons before the flash excitation. Those results were consistent with the notion that protons released by the redox reactions were first delivered to the protonatable domain groups before any protons were delivered to the CF₀-CF₁ complex. However, it could not be strictly ruled out that during the approximately 20–30 flash lag in the onset of ATP formation there was either: (a) a parallel filling of two pools, one on the main energization route (the lumen according to the original chemiosmotic hypothesis (13, 14)) and the other on a side path (consisting of the identifiable domains); or (b) the sequestered domains were in series with the lumen such that protons might obligatorily fill emptied domains but then proceed via the lumen to the CF₀-CF₁.

We suggest that a robust test for the mode of involvement or the noninvolvement of the sequestered domain protons in ATP formation is available by combining three controllable experimental variables: (i) poise thylakoids with the sequestered domains either fully protonated or depleted of protons; (ii) use the electrical potential (Δψ) component of the proton-motive force to achieve rapid energization of ATP formation rather than relying only on the more slowly developing ΔpH."
contribution; and (iii) apply (i) and (ii) to thylakoids pre-treated so as to show either delocalized or localized proton gradient coupling (15, 17). The rationale for this tripartite experiment is described below.

Item (i) was mentioned above. Part (ii) involves the $\Delta \psi$, which is known to contribute to the rapid onset of ATP formation in flashing light (18, 19) and in dark conditions when the $\Delta \psi$ is provided by an electric field imposed across the sample (20, 21) or by addition of $K^+$ and valinomycin (22-24) to thylakoids that have some $\Delta \phi$ across the membrane (either in a postillumination experiment (22, 23) or an acid-base jump protocol (24)). In the experiments reported here, we will use the redox turnovers to provide the $\Delta \psi$ (18, 19).

Item (iii) is a recent development from this laboratory which showed that storing thylakoids in a low salt 200 mM sucrose-containing buffer gave localized proton gradient responses (Ref. 15, but as previously reported (19)), but storage in buffer containing 100 mM KCl (in place of the sucrose) gave a clearly measurable delocalized coupling response (15, 17). The criteria for judging localized or delocalized responses was whether permeable buffers such as pyridine affected either the length of the lag in energization for ATP formation or yield of postillumination phosphorylation in the way predicted for delocalized proton gradient coupling (12, 15, 17, 25).

We repeated the experiments of Dilley and Schreiber (10), but this time valinomycin was omitted so that the proton-motive force would be dominated by the $\Delta \psi$ at the onset of ATP synthesis. In fully dark-adapted thylakoids ATP formation typically begins before the third or fourth flash when $\Delta \psi$ can contribute and is retarded until 12-15 flashes when valinomycin and $K^+$ are present to collapse the $\Delta \psi$ (10, 19, 25). We compared the ATP onset flash lags in samples stored either in the "low salt"- or "high salt"-containing buffer (the phosphorylation reaction is of identical composition in all cases) either with the sequestered domains in the normal fully protonated state or depleted of protons by the reversible uncoupling treatment. To accomplish this we relied on three experimental observations. First, we know that the sequestered domains in chloroplasts are normally prepared them are fully protonated (5). Second, the half-time for passive proton permeability of the thylakoid membrane to protons is no more than 30 s at 10 °C (26). Thus, if thylakoids are incubated in the dark for at least 3 min, proton equilibration between the lumen and the external phase would be >98% complete (cf. "Discussion"). Third, Dilley and Schreiber (10) showed that thylakoids can be reversibly uncoupled by adding BSA to a suspension containing CCCP; apparently BSA binds the CCCP and functionally removes it from solution. This technique can be used to set the domains of thylakoids suspended at alkaline pH into either an unprotonated (CCCP added, then BSA) or protonated (BSA added, then CCCP) state. In either case, the dark incubation period is chosen to be long enough to ensure that protons in the lumen have come to equilibrium with those in the external medium. The electric field established upon illumination causes protons to be electrrophoresed into the CF$_{0}$-CF$_{1}$ complex, and it is expected that those protons with the easiest access to the CF$_{0}$ portion of the coupling factor will be the ones involved in initiation of ATP synthesis.

We predicted that, with the "low salt-stored" thylakoids (which show localized coupling responses), if the sequestered domains are on a side path not involved with the events of proton diffusion to the CF$_{0}$-CF$_{1}$, then there should be no difference in the flash lags for the onset of ATP formation driven by the $\Delta \psi$ component of the $\Delta \phi$ between the control (domains initially protonated) and reversibly uncoupled (domains initially unprotonated) samples. This is because, by that point of view, the domains are assumed not to be involved in energy-linked proton fluxes, and the $\Delta \phi$ should have acted on other protons that are "in line" for diffusion into the CF$_{0}$. In that scheme the proton concentration at the lumen entrance of the CF$_{0}$ is assumed to be the same in both samples. Similarly, if we take the view that the domains are involved with the H$^+$ diffusion pathway but are in series with the lumen, into which the domains deposit the protons, then the $\Delta \psi$ should still initiate ATP formation at the same flash number, regardless of the status of the domain protonation-deprotonation state, because the lumen and the CF$_{0}$ environment should be at the same initial pH.

On the other hand, if the sequestered domain protons are the next in line on a diffusion pathway into the CF$_{0}$-CF$_{1}$, then the onset of ATP formation would be delayed when the domains had been emptied of protons by a reversible uncoupling treatment before initiation of the flash sequence, because the proton concentration at the putative domain entrance to the CF$_{0}$ would be lower than in the control. In such an experiment, electron transport subsequent to depleting the domain proton release sites should protrude the domain buffering groups, restoring the short lags observed with the controls.

With the "high salt-stored" thylakoids, which have a delocalized proton gradient energy coupling pattern, the proton release sites should be in equilibrium with the lumen, and protons in the lumen should have access to the CF$_{0}$, H$^+$ channel, so the reversible uncoupler treatment should not result in a different ATP onset lag number.

Finally, since other known effects of domain protons have only been detectable at alkaline pH (3-7, 9, 10, 27), we would expect (for the low salt-stored case) the reversible uncoupling treatment to extend the onset lags only at high pH.

MATERIALS AND METHODS

Chloroplasts were isolated from growth chamber spinach as described in Ref. 9. In part of the experiments 5 mM DTT was included in the grinding and resuspension media, but a comparison of the results indicated that it had no effect on the ATP formation onset lag parameters measured in these experiments. Two types of thylakoid suspensions were used, termed "low salt-stored" or "high salt-stored." The low salt storage medium was 200 mM sucrose, 2 mM MgCl$_2$, and 0.5 mg/ml BSA. Thylakoids kept under low KCl conditions show localized proton gradient coupling characteristics by the criteria of permeable buffer (pyridine) effects on the length of the ATP formation onset lag and on the postillumination phosphorylation yield (15, 25).

The high salt-stored thylakoids were prepared similarly except that the 200 mM sucrose in the suspension medium was replaced by 30 mM sucrose and 100 mM KCl. Those thylakoids show delocalized proton gradient coupling responses as previously documented (15, 17). The thylakoids from either preparation were diluted about 200-fold into the identical phosphorylation assay media specified below, making only slight changes in the composition of the phosphorylation reaction medium.

All reactions were performed at 10 °C. Assays for flash-induced ATP synthesis were performed as described in Ref. 15. Some light from the flash lamp leaks through the cutoff filter pair to the photomultiplier tube, causing a spike which serves as an event marker. The periodicity observed in the spike heights was introduced as the digitizer caught the analog signal at different points in the rise and fall of the spike, and is purely artificial. No such periodicity is observed at the base line of the digitized signal, nor was it present in the analog signals which were always recorded simultaneously (on a strip chart recorder). The flash lag parameters were determined from the strip chart recorder tracings of the analog signal, thus avoiding the digitizer-induced periodicity. See Fig. 1 of Ref. 10 for a representation of the analog signal.

Unless otherwise indicated, chloroplasts were suspended at 15 μg Chl/ml in 0.8 ml of assay medium containing 50 mM sodium Tricine, pH 8.5, 3 mM MgCl$_2$, 1 mM KH$_2$PO$_4$, 5 mM DTT, 0.1 mM methyl viologen, 5 or 10 μM diadenosine pentaphosphate, 0.1 mM ADP, and
10 µl of luciferin-luciferase/ml (see below). When present, the concentrations of BSA, CCCP, and valinomycin were 1.0 mg/ml, 0.4 µM, and 0.4 µM, respectively. The buffers used in Figs. 6 and 7 were Tricine at pH ≥ 7.7 and PIPES at pH ≤ 7.3. For all other experiments, Tricine was used at pH ≥ 8.0 and MOPS at pH 7.0 and 6.9. Flash-induced absorbance changes at 515 nm were measured in the assay media described above but without luciferin-luciferase; in Fig. 2, ADP, diadenosine pentaphosphate, and DTT were also omitted.

The flash lags for ATP synthesis were determined by estimating the first detectable rise of the signal from the base line, and the second number to the point of crossing of the extrapolated steady-state line. Both criteria estimates are given in the figures for each trace, separated by a colon. The criterion used for the lag determination does not change the conclusions drawn from the presented experiments.

All reagents were purchased from commercial vendors. ADP was purified from contaminating ATP by passage over a Dowex 1-X4 ion exchange column (28). Luciferin-luciferase was purchased from LKB (1243-102 ATP monitoring kit). Stock solutions of the luciferin-luciferase reagent were prepared by adding 1.5 ml of distilled water to the contents of a vial and freezing aliquots for later use.

RESULTS

Effects of Reversible Uncoupling on the ATP Onset Flash Lag with "Low Salt-treated" Thylakoids at Alkaline pH—As pointed out in the Introduction, protons sequestered in the domains can be equilibrated with those in the bulk phases by CCCP. A subsequent addition of BSA removes the CCCP and recouples the chloroplasts (10). We used this technique to compare the onset flash lag for ATP synthesis in samples starting with domains either fully protonated ("BSA only") or "BSA, then CCCP" or deprotonated ("CCCP, then BSA"). One such experiment performed at pH 8.5 is shown in Fig. 1. In traces b, c, g, and h, the first compound (BSA or CCCP) was present when the chloroplasts were added to the assay buffer, and the second compound (CCCP or BSA) was added 30 s later. It can be seen that for the dark-adapted samples (left-hand side), the reversibly uncoupled thylakoids (trace c) required 12 more flashes than did the controls (traces a and b) to reach the threshold for ATP synthesis. That a Δψ was the major contributor to the Δt at the onset of ATP formation can be seen by comparing traces c-a to the 26-flash lag observed when valinomycin was added (trace d). Comparison of the high flash yields of ATP formation observed in traces b and c, 0.6-0.7 nmol of ATP (mg Chl flash)−1, with that in trace e when only CCCP was added, 0.38 nmol ATP (mg Chl flash)−1, indicates that BSA was able to completely withdraw the CCCP from solution. The low amount of ATP made in the presence of CCCP alone (trace e) was probably due in part to the low CCCP concentration used (400 nM) and perhaps to a slow adsorption of the CCCP to the BSA present in the stock luciferin-luciferase reagent; this "endogenous" BSA was always present at ≤0.1 mg/ml.

If the loss of protons from the domains were the cause of the extended lag in trace c ("CCCP, then BSA"), then a brief preillumination subsequent to the reversible uncoupling treatment should restore the short lags by reprotinating the domain buffering groups. This experiment is shown on the right side of Fig. 1 using the same chloroplast preparation as used on the left. The thylakoids were subjected to reversible uncoupling (trace h) or control (traces f and g) conditions between t = 0 and t = 30 s and then preilluminated for 10 s at t = 1 min. The flash trains were initiated after an additional 4-min dark adaptation period. All three samples required the same number of flashes to initiate photophosphorylation, demonstrating that the effects of reversible uncoupling on the flash lag were reversed by the brief burst of electron transport. It is noteworthy that no ADP was present during the preillumination period, eliminating the possibility that a dark ΔpH was maintained by ATPase activity prior to the flash train. The absence of ATPase activity in the dark period prior to the flash sequence is demonstrated by the nonsloping base lines of the traces on the right-hand side of Fig. 1, prior to starting the flash sequences.

As few as 15 single-turnover red light flashes given to thylakoids after the CCCP-BSA reversible uncoupling treatment were as effective as the 10 s of red light used for the Fig. 1 experiment (Table I).

The extension of the ATP onset flash lag by reversible uncoupling observed in Fig. 1 might be explained by postulating that the removal of CCCP from the chloroplast suspension by BSA was time- and/or light-dependent. If so, the extra flashes required before the onset of ATP formation by other reversible uncoupling would simply reflect such a time or light requirement for the binding of CCCP by BSA. This possibility can be ruled out if it can be established that CCCP had indeed been removed from solution by the time the first flash in the sequence was fired.

Fig. 2 shows the decay of the Δψ-indicating absorbance change at 515 nm observed by averaging the signals from only four flashes under conditions similar to those used in the experiment of Fig. 1. Compared to the control samples ("no additions" and "BSA only"), the addition of the protonophore CCCP caused the expected acceleration of the decay of the flash-induced electric field. When BSA was added to the sample exposed to CCCP for 30 s in the dark, the electric field decayed with kinetics indistinguishable from those of the controls. Ideally this experiment would have been performed without signal averaging four samples so that the decay of Δψ in a single flash would have been monitored. However, this is beyond the resolution of our instrument. In order to achieve a reasonable signal to noise ratio, we averaged the signals from four flashes separated by 10 s. This protocol very nearly simulates a single-flash experiment and kept us well below the number of extra flashes required for the onset of ATP.

\[ \text{Dual Energy Coupling Pathways in Chloroplasts} \]
that the initial ApH imposed by transferring the chloroplasts adsorbed by the added BSA at the time the first flash was fired in the experiment of Fig. 1 except that a sequence of 130 flashes was delivered at 5 Hz at t = 5.5, and a second cycle of flashes was delivered at t = 5.6 min instead of 10 s of red light.

synthesis after reversible uncoupling. Since the control and reversibly uncoupled samples produced the same ΔpH decay kinetics, we can conclude that the CCCP had been largely adsorbed by the added BSA at the time the first flash was fired in the experiment of Fig. 1.

Another trivial explanation for the data in Fig. 1 could be that the initial ΔpH imposed by transferring the chloroplasts from pH 7.5 storage media into the pH 8.5 assay medium remained high enough after 5 min to affect the ATP onset flash lag. This appears unlikely because the passive proton permeability of the thylakoid membrane allows protons to cross with a half-time of less than 30 s at pH 8.5 and 10 °C (26). Hence, proton equilibration from the lumen to the external phase would be >99.9% complete in the 5-min interval between chloroplast addition and the first flash. Although we have confidence in the validity of this argument, we also tested experimentally for effects of any residual transfer-imposed ΔpH, and the results are reported in Figs. 3 and 4.

If the onset lag is related to a residual ΔpH induced by transferring the chloroplasts into the assay medium, then the lag should become longer if the lumen protons are given more time to equilibrate with the alkaline external medium before the flash sequence is initiated. Fig. 3 shows that neither the lag nor the ATP yield/flash was significantly changed by extending the time between chloroplast addition and initiation of the flash sequence from 3 to 9 min. In the experiment depicted in Fig. 4, the chloroplasts were resuspended in pH 8.5 medium at the time of their preparation and stored on ice for >90 min before use. Thus, no ΔpH was imposed by transferring the chloroplasts from their storage to assay media. Compared to the low pH control, the flash ATP yields were lower and the onset lags were longer due to this prolonged incubation at high pH. Nonetheless, we were still able to observe a flash lag extension after reversible uncoupling with the chloroplasts stored at alkaline pH.

The experiments from Figs. 3 and 4 demonstrate that the transient proton gradient imposed by adding thylakoids stored at low pH to high pH assay buffer cannot account for the extension of the flash lag by reversible uncoupling observed in Fig. 1.

### Table I

**Preillumination with 15 single-turnover flashes is sufficient to refill previously dumped domains in “low salt-stored” thylakoids**

The effect of CI-CCCP and BSA on the ATP onset flash lag with chloroplasts resuspended in high salt or low salt storage media is shown below. Chloroplasts were prepared as described under "Materials and Methods" either in low salt storage media or in high salt media in which 200 mM sucrose was replaced by 100 mM KCl and 30 mM sucrose. Conditions are as in Fig. 1 except that a sequence of 130 flashes was delivered at 5 Hz at t = 5.5, and a second cycle of flashes was delivered at t = 5.6 min instead of 10 s of red light.

| Treatments | Preillumination | Number of flashes to ATP formation onset | ATP yield/flash (nmol ATP (mg Chl-flash)^-1) |
|------------|----------------|-----------------------------------------|---------------------------------------------|
| 1. BSA, then CCCP | No | 13 ± 0.25 ± 0 | 0.71 ± 0.08 |
| 2. CCCP, then BSA | No | 21 ± 1.34 ± 1 | 0.65 ± 0.09 |
| 3. CCCP, then BSA, then 14 flashes | 14 flashes | 13 ± 1.25 ± 1 | 0.39 ± 0.06 |
| 4. CCCP, then BSA, then 15 flashes | 15 flashes | 11 ± 1.25 ± 1 | 0.62 ± 0.02 |
| 5. CCCP, then BSA, then 20 flashes | 20 flashes | 10 ± 2.22 ± 0 | 0.63 ± 0.02 |

### Fig. 3.

**Effect of incubation time on the ATP onset flash lag.** Chloroplasts were added to pH 8.5 medium containing BSA at t = 0 min and incubated in the dark for the times indicated. Luciferin-luciferase was added 60 s before initiation of the sequence of 90 flashes delivered at 5 Hz. y and t are defined in the legend to Fig. 1.
that the proton gradient established in high salt-treated thylakoids is completely delocalized (15, 16, 25). Thus, when a $\Delta\psi$ force is applied, protons in the lumen should have first access to the CF$\alpha$-CF$\beta$ complex. In that model, it is expected that the ATP onset lag length should not be influenced by the domain depletion treatment. A different result might be predicted for the case where only the $\Delta\phi$ contribution to the protonmotive force is allowed to build up, but other experiments, to be reported later, will address that question. As a control, on the same days experiments were run with low salt-treated thylakoids, and the lag extensions caused by the reversible uncoupling treatment were 8:12 flashes, clearly significantly different from the high salt-treated cases with standard deviations of at most $\pm 2$ flashes (part B, lines 3 and 4 or 5 and 6).

Valuable information concerning the role of domain protons can be obtained from comparing the ATP onset lag in a first cycle of flashes with the lag observed in a second cycle following a 3–4-min dark period. Earlier results had shown that CCCP-BSA reversible uncoupling treatment extended the ATP onset lag for the first flash cycle by about 10 flashes more than the control, but on a second cycle (after a 4–8-min dark period) the onset lag was shortened back to near the control value (10). That was considered as evidence that the sequestered domains were required to be in the fully protonated state before any protons were available for efflux through the CF$\alpha$-CF$\beta$. However, those experiments were done with thylakoids prepared similarly to our low salt-stored samples.

In the present experiments, using high salt-stored thylakoids, the second cycle flash sequence gave the same ATP onset flash lag lengths as the first flash sequence for the domain depletion treatment (CCCP first, lines 4 or 6, Table II) as for the non-depleted control (BSA first, lines 3 or 5). These data agree with the first cycle results and provide further evidence that the first protons through the CF$\alpha$-CF$\beta$ complex driven by the $\Delta\phi$ force in the high salt-treated case are those in the lumen. Perhaps second cycle protonation events may occur in the sequestered domains, but they have no influence on the initial $\Delta\phi$-driven proton fluxes giving energization of ATP formation.

The situation for the second cycle of energization was very different with low salt-stored thylakoids but in agreement with the earlier results (10). After depleting the sequestered domain with CCCP added before BSA, and observing a 8 or 12 flash lag increase (from 14:26 out to 22:38 flashes on the first cycle, Table II, line 6 compared with line 5), the second cycle showed the effect of refilling the depleted domains and the lag was shortened back to 11:16 flashes (line 6, 2nd cycle), close to the 12:18 flash lag observed with the nondepleted control (line 5). The interpretation is that when the domains can keep the energization proton fluxes localized, as we believe

### Table II

**Effect of sequestered domain dumping treatment on ATP onset lag parameters in 100 mM KCl-treated (high salt) compared to low salt-treated thylakoids**

The effect of preilluminating flashes on the ATP onset flash lag with chloroplasts resuspended in low or high salt storage media is shown below. Conditions are described as in Fig. 1 except that a specified number of preilluminating flashes was delivered at 5 Hz to $t = 1$ min instead of 10 s of red light, and at $t = 5.5$ min a sequence of 130 flashes at 5 Hz started.

| Treatments (additions before flashes) | Number of flashes to ATP formation onset | ATP yield/flash |
|--------------------------------------|-----------------------------------------|----------------|
|                                      | 1st cycle | 2nd cycle | 1st cycle | 2nd cycle |
|                                      |           |           |           |           |
| A. High salt storage                 |           |           |           |           |
| 1. BSA only                         | 7:20      |           | 0.60      |           |
| 2. CCCP only                        | 19:41     | 0.58      |           |           |
| 3. BSA, then CCCP                   | 13±1:29±1 | 10±0:22±1 | 0.55±0.06 | 0.53±0.05 |
| 4. CCCP, then BSA                   | 13±2:31±1 | 13±1:27±1 | 0.58±0.00 | 0.57±0.01 |
| 5. BSA, then CCCP*                  | 13:28     | 10:27     | 0.59      | 0.59      |
| 6. CCCP, then BSA*                  | 13±1:33±1 | 10±3:25±2 | 0.50±0.08 | 0.47±0.06 |
| 7. BSA, valinomycin*                | 39±1:49±1 | 36±2:43±1 | 0.43±1    | 0.40±0.03 |

| B. Low salt storage                 |           |           |           |           |
| 1. BSA only                         | 8:20      | 0.70      |           |           |
| 2. CCCP only                        | 27:44     | 0.57      |           |           |
| 3. BSA, then CCCP                   | 11:25     | 0.69      |           |           |
| 4. CCCP, then BSA                   | 15±1:37±1 |           | 0.56±0.06 |           |
| 5. BSA, then CCCP*                  | 14±0:26±1 | 12±2:18±1 | 0.59±0.03 | 0.52±0.12 |
| 6. CCCP, then BSA*                  | 22±4:38±2 | 11±2:16±1 | 0.44±0.09 | 0.40±0.07 |

* Experiments from a different day.
* 400 mM valinomycin added.
is the case with the low salt-stored thylakoids, the proton depleted domains are refilled during a first cycle of flashes, and the reprotonated domains permit a shorter energization onset lag on a subsequent second cycle of flashes.

Effects of Reversible Uncoupling on the ATP Onset Flash Lag at Neutral pH—In other experimental systems, effects of domain-sequestered protons detected by uncoupler addition to dark-adapted thylakoids have been observed only at alkaline pH (3–7, 9, 10, 27). This can be explained if it is postulated that dark-held thylakoids maintain for tens of minutes a pH of approximately 7 in the domains regardless of the more alkaline pH of the suspending medium (5, 7). At pH 7.0 in the assay medium, domain protons would be at the same concentration as those in the external phases and consequently would not move in response to uncoupler addition.

If, as postulated, the protonation state of the special domains controlled the length of the ATP onset flash lag in Fig. 1, reversible uncoupling would not be expected to cause an extension of the ATP onset flash lag at pH 7.0. This was tested in the experiment shown in Fig. 5 and, indeed, the same lag was observed with the (“BSA, then CCCP” and “CCCP, then BSA” samples. Both lags were somewhat longer then seen in the “BSA only” control but were significantly shorter than observed with the “BSA and valinomycin” or “CCCP only” samples.

Effects of pH on the ATP Onset Flash Lag—Although it is not readily apparent from comparison of Figs. 1 and 5, we generally found that the ATP onset flash lag was considerably shorter at alkaline than at neutral pH in our reversible uncoupling experiments (see Figs. 3 and 4 for more typical lags at pH 8.4–8.5). When valinomycin and K+ kept the Δψ suppressed, we also have found longer lags at pH 7.0 than at 8, but that was shown to be due to a slower turnover (or activation) of the coupling mechanism at the lower pH (25). At flash frequencies <1 Hz, there was no difference in the onset lags. Nonetheless, for the case with Δψ contributing to the energization, it could be expected that CF₁ activation should be faster, so we wondered if an artifact had been introduced by the presence of CCCP and BSA, in either order of addition. Thus, we examined more closely the pH depend-

tence of the ATP onset lags and flash yields when neither compound was present. Fig. 6 demonstrates that, even in the absence of CCCP and BSA, the onset lags (triangles) were greater at lower pH values of the assay medium. The ATP flash yields (circles) were also sensitive to pH but to a lesser extent than were the lags. The possibility that the flash-induced electric field was smaller at pH 7 than at pH 8.5 was eliminated by the experiment shown in Fig. 7. We found that, if anything, the magnitude of the Δψ increased and the decay rate decreased when the pH was lowered.

The Effects of Reversible Uncoupling on the ATP Onset Flash Lag Are Not Due to Coupling Factor Activation—It was not clear in our earlier experiments (Fig. 1 and Ref. 10) whether the flash lag extension induced by reversible uncoupling was due to a requirement for domain protons to overcome the thermodynamic threshold for ATP synthesis or to activate the CF₀-CF₁ complex to a state in which it can synthesize ATP (cf. Ref. 21). To answer this question, we examined the effects of reversible uncoupling on samples in which the coupling factors had been activated by preillumination in the presence of DTT (29). In the experiment of Fig. 8, we illuminated the thylakoids for 2 min with saturating light intensity in the presence of DTT starting 1 min (t = 1 min) after chloroplast addition to the assay medium. At t = 6 min, the reversible uncoupling treatment was performed, and at t = 8 min, either ATP was added to test for ATPase activity (upper three traces) or flashes were initiated to determine the lag and yield parameters (lower two traces). It can be seen that considerable ATPase activity remained at t = 8 min in both the control and reversibly uncoupled samples, demonstrating that the reversible uncoupling treatment had no effect on the degree of activation of the coupling factor. Conversely, DTT activation had no effect on the flash lag extension due

![Fig. 5. Effect of reversible uncoupling on the ATP onset flash lags at neutral pH. Chloroplasts were assayed in pH 7.0 buffer using the 3-min protocol as in Fig. 4. Ninety flashes were given at 2 Hz. val, γ, and I are defined in the legend to Fig. 1.](image)

![Fig. 6. The pH dependence of ATP flash lags and flash yields in the absence of BSA and CCCP. Chloroplasts were transferred to the assay medium 3 min before the sequence of 90 flashes at 2 Hz was initiated.](image)

![Fig. 7. Measurement of the 515-nm electrochromic absorption band shift at pH 8.45 and 6.9. Conditions were as described under "Materials and Methods." Chloroplasts were incubated for 2 min in the assay buffers before data acquisition was initiated. Traces are the averaged signals obtained with 16 flashes delivered at 1/8 Hz.](image)
to removal of the protons from the special domains. Therefore, we can conclude that the domain protons are not merely involved in setting the coupling factor to a state analogous to the activated state achieved by thiol plus light activation.

**DISCUSSION**

Interpreting Control Experiments—It is clear that in the low salt-stored thylakoids having localized proton gradient responses the protonation state of the sequestered domains greatly influenced the length of the ATP formation onset lag. In contrast, high salt-stored thylakoids having delocalized energy coupling responses had the same Δψ-dependent energization onset lag with or without the sequestered domain proton depletion treatment. The simplest interpretation is that the protons in the sequestered domains are the ones having first access to the CF, in the low salt-stored sample. In contrast, when the proton-motive force is established in a delocalized mode, as is the case for the high salt-treated thylakoids, the Δψ-driven ATP formation onset lag is unaffected by the reversible uncoupler treatment, as expected if luminal protons are the ones having first access to the CF. An alternative view, that in all cases the proton pathway to the CF includes an obligatory transit through the lumen, does not seem to us a reasonable interpretation. Further discussion of this will be taken up below after mention of certain control experiments.

This experiment would not be valid if there were significant differences between the two thylakoid samples, such as high KCl-induced swelling, changes in the extent or decay kinetics of the electric field indicating 515-nm absorbance change, or different electron or proton transport/flash. Those and other controls have been checked (17), and there were no detectable differences in those parameters between the low and high salt-treated samples (assayed in the phosphorylation assay media, which was always identical).

The above argument for the low salt-treated thylakoid case is dependent on the assumption that with or without the reversible uncoupling treatment the protons in the lumen had equilibrated with those in the external medium at the time the flash train was initiated. A number of published experiments supports that assumption. First, as discussed above, the half-time for passive proton diffusion across the thylakoid membrane is <30 s at 10 °C (26). This diffusion time rules out the possibility that lumen protons are held out of equilibrium with the external phase by the membrane for many minutes when chloroplasts are kept in the dark. The experiments on the left-hand side of Fig. 1 were performed after a 5-min incubation period in the dark following transfer of the samples to the assay medium, a time sufficient to allow >99.9% equilibration of lumen protons. Second, both Laszlo et al. (3) and Graan et al. (19) have used the distribution of methylamine (after 10 or more min of incubation) in response to a pH gradient to measure the transmembrane ΔpH in dark-adapted thylakoids. While both groups measured close to a non-zero ΔpH (slightly acidic, 0.2-0.5 pH units lower in the lumen), it was not altered by the addition of uncouplers, indicating that the higher internal concentration of amine was probably due to its binding at the membrane surface and not to a true pH gradient. In recent ΔpH determinations with 3- and 5-min incubations, similar to the time frame for doing the experiments reported here, an uncoupler-sensitive ΔpH of near 0.2 pH unit was detected. In view of the other arguments discounting a ΔpH as the origin of the reversible uncoupling-induced change in the onset lag, it seems unlikely that the measured 0.2 ΔpH present at 3-5 min was a significant factor in the data pattern. Third, Hangarter and Good (24) measured ATP synthesis in response to imposed pH and salt jumps in chloroplasts dark-adapted in the first stage for 3 min. They noted a strict additivity of the Δψ and ΔpH components of the proton-motive force with respect to ATP synthesis. It seems unlikely that such an additivity could have been observed had they continuously underestimated the pH of the lumen before the addition of the alkaline second stage buffer.

In addition to relying on these published experiments, we performed two more control experiments to address whether our results from Fig. 1 and Table I could have been due to the presence of a residual ΔpH that arose from the transfer of the thylakoids from low pH storage medium to high pH assay medium. The results, presented in Figs. 3 and 4, ruled out this trivial explanation.

It has been repeatedly postulated that the coupling factor must undergo a change from an inactive state, found in the dark, to an active state in the light, in order to catalyze ATP synthesis (see Ref. 20 for a thorough discussion of this point). Recently, Hangarter and Ort (26) determined that at least some of the ΔpH required to initiate ATP formation in flashing light was required to activate the coupling factor. It seemed possible that the additional flash lag for ATP synthesis we observed after reversible uncoupling could have been due to an uncoupler-induced requirement for an additional activating proton flux (30) through the coupling factor. To examine this possibility, we performed the reversible uncoupling experiments on samples in which the coupling factors had previously been activated by DTT and 2 min of light (29) (Fig. 8). We found no evidence suggesting either that DTT activation of the CF,·CF complex eliminated the requirement for the presence of domain-associated protons for initiation of Δψ-driven ATP synthesis or that the activation state of the enzyme complex was affected by the protonation state of the domains.

---

[1] F. C. T. Allnutt and R. A. Dilley, work in progress.
Hypotheses and Speculation—The results with the high salt-treated thylakoids are consistent with the hypothesis discussed in the Introduction, namely that a delocalized proton gradient coupling mode should give that pattern, if protons in the lumen are the “first through” the CF$_6$ under the development of the $\Delta$$V$-dominated proton-motive force. The problem of interpretation then becomes one of accounting for the results observed in the low salt-treated thylakoid case.

The quite distinct onset lag responses of the two thylakoid types to the domain-proton depletion treatment is consistent with the hypothesis that in thylakoids showing the localized coupling responses by the permeable buffer effect criteria, the protons in the domains are en route to the CF$_6$, without the lumen being an obligatory part of the diffusion pathway. An alternative model, more in keeping with traditional chemiosmotic theory, depicts the proton diffusion pathway as domain $\rightarrow$ lumen $\rightarrow$ CF$_{6}$-$\text{CF}_1$. This alternative nicely explains the results obtained here with the high salt-treated thylakoids, and hence it certainly should not be lightly dismissed as also fitting the data for the low salt-treated case. We assert that the alternative model would have been a more viable interpretation for the low salt case, were it not for the greatly contrasting results observed here with the high salt-treated thylakoids compared to the low salt case.

Marshalling the evidence and arguments for the more speculative model that the low salt-treated thylakoids can (but are not necessarily constrained to) utilize a strictly localized H$^+$ diffusion pathway, the following items can be mentioned.

(a) A localized proton gradient coupling behavior has been strongly suggested for the low salt-treated thylakoids from permeable buffer effects on the ATP onset lag and post-illumination phosphorylation after a flash train in which ATP formation occurs (12, 15, 17, 19). The reversible shifting from a localized to a delocalized pattern and back again to the localized mode by controlling the salt concentrations (16, 17) is evidence that the effects are not due to irreversible salt-induced membrane changes or degradation effects, but due to a regulated membrane response. Moreover, it has been clearly shown that the low salt-treated thylakoids under similar energization conditions (except that ADP and P$_i$ were not added until after the last flash of a sequence) gave a delocalized coupling pattern when the post-illumination phosphorylation mode was of the traditional type (12). Those results were discussed in terms of the existence of two modes of proton gradient energy coupling, either delocalized or localized, depending on conditions such as ionic environment (17). The concept that we propose is that protons in the special domains can be utilized via strictly localized gradient coupling provided that: (i) the ATP formation steps are able to proceed sufficiently rapidly to utilize the proton-motive force initially established in the domains, so as to keep the proton back pressure low enough to not saturate the buffering capacity of the sequestered domains (12, 17); and (ii) the ionic balances, particularly the divalent cations, are such as to maintain a proposed gating function in the localized mode. Calcium ions in particular seem to be critically involved in regulating the localized $\rightarrow$ delocalized gating action (16).

(c) The question can be raised as to why there is perhaps a 4-5-flash lag in the absence of a domain dumping treatment, i.e. why shouldn’t ATP formation begin on the first or second flash? It should be kept in mind that the metastable buffering pool is expected to be slowly emptied when the pH is higher than the pK$_1$ ($\approx$7.5-7.8). Fig. 4 shows this effect due to storage at pH 8.5 compared to pH 7.5. That is, there usually may be a certain, but variable, amount of partially discharged domains that absorb protons before being fully protonated.

A similar question is why there is often a gradual, rather than an abrupt, onset of full efficiency ATP formation/flash (cf. Fig. 1). A localized proton mechanism could, in principle, work with low buffering capacity in the pathway. However, it should be pointed out that the localized coupling phenomenon occurs with a threshold buffering capacity (for $\Delta$H-driven ATP formation) of near 60 nmol H$^+$/mg Chl$^{-1}$, requiring 15-20 flashes to fill (12, 26). Thus, it is reasonable to expect that there might be a distribution of ATP onset lags in different thylakoids of a sample. Another cause of a gradual attainment of the maximum ATP yield/flash may be due to the “bringing on line” the full compliment of activated CF$_6$ complexes. Graber et al. (32) showed that the full activation of the CF$_6$ population does not occur simultaneously, but activation is characterized by a rapid onset of about 20% of the CF$_6$ units followed by a slower activation of the remaining units. That explanation is consistent with the data of Fig. 8d, where in those light and DTT-activated thylakoids there was a more abrupt attainment of the steady state yield/flash than, say, in the data for Fig. 1, a or b, where the thylakoids were not preactivated with light and DTT. An expression of the gradualness in the attainment of full efficiency is in the difference between the first and second onset lag parameters, 6:10 for Fig. 8d and 4:18 or 8:21 for Fig. 1, a and b, respectively.

Another reason for the less than abrupt attainment of the full efficiency yield/flash may be due to the fact that 2-Hz flashes were given, well below the rate of maximal turnover of the redox centers. That may accentuate whatever lag due to pool filling there may be as the long (0.5 s) dark times may allow a finite amount of H$^+$ efflux during the lag phase.

It is significant that the buffering capacity of the pK$_1$ = 7.5 sequestered amine buffering domain is about 30-40 nmol H$^+$/mg Chl$^{-1}$, closely matching the amount of the amine array (1, 2, 31). Moreover, the pK$_1$ ($\approx$7.5) domains are dumped before the flashes are given (10). Thus, we view the first 15-20 flashes given in the former circumstances as being required to fill the low pK$^-$ (6.5) buffering groups. When $\Delta$$V$ can contribute to the energization, the $\Delta$H need not develop to as great an extent, implying that the low pK array need not be protonated before the energization threshold is reached. This is reasonable if the $\Delta$$V$ can act on the protons, electrophoresing them out through the CF$_6$-$\text{CF}_1$ (14). The higher pK localized domains, however, must be protonated before the threshold energization is reached, as indicated by the results presented here.
(d) The question must be raised of why, at pH 7 compared to pH 8, more flashes are required to reach the energetic threshold and there is a lower ATP yield/flash. This could be expected in the absence of a $\Delta \psi^*$ contribution because at pH 7 about 10-fold more protons must be accumulated than at pH 8 to develop the equivalent increase in the $\Delta \psi$ part of the proton-motive force. Either the delocalized or the localized models are consistent with this, however, when we consider the recent evidence (12) that the localized domains have a significant buffering capacity, not all that different from that of the lumen. Furthermore, the buffering capacity is considerably greater as the pH approaches 5.5 and lower, due to the predominant carboxyl component to the buffering (34), so in that range more proton accumulation must occur to cause a given drop in pH. Therefore, for either thylakoid membrane coupling mode, a similar pattern of increased flashes could be predicted for pH 7 compared to pH 8 conditions.

However, the above points may be irrelevant because, in the case of the Fig. 6 data, the $\Delta \psi^*$ component contributed to the energization, and the lumen or the special domain pH does not have to be so acidic as in the case where $\Delta \psi$ is suppressed. Yet in 20–30 flashes, the observed lag in the pH 6.5–7.0 experiments, a considerable drop in pH is expected even with a $\Delta \psi^*$ contribution, indicating that other factors than just the proton-motive force are involved. It seems likely that the long onset lag at pH $\approx$ 7 is due to slower CF activation and/or slower turnover time than at pH 8. The length of the ATP onset flash lag was found to be a function of the flash frequency at pH 7.0 but not at pH 8.0 (25). At sufficiently slow flash rates, the lags were similar for the two pH values, indicating that the longer lags probably reflect a slower turnover time of the complex at the lower pH. Other experiments showed that CF activation, measured by debridging of tightly bound $[^{14}C]$ADP, correlated well with a component of $H^+$ efflux through the CF, and was much slower at pH 7.0 than at pH 8.0 (30).

(e) Given (a) through (d), the lag extension induced by the sequestered domain damping treatment for the low salt-treated case but its absence in the high salt-treated case is consistent with the concept that protons in the localized domains are driven into the CF$_{\text{P}}$–CF$_{\text{O}}$ via a pathway that does not include the lumen. If the lumen were an obligatory part of the proton diffusion pathway in the low salt-treated case, as seems to be the case for the high salt-treated thylakoids, then it is difficult to explain why this difference in the pattern of effects on the ATP formation onset lag length in the two thylakoid types should occur after the domain damping treatment. This seems to be an especially germane point for the case when the $\Delta \psi^*$ part of the proton-motive force is permitted to contribute to the energization, i.e. if the bulk lumen phase is always the immediate origin of the electrophoresed protons into the CF$_{\text{O}}$, why shouldn’t the lag responses be similar in both types of thylakoids?

The above arguments summarizing other work along with the present results are consistent with the hypothesis that thylakoids have dual proton gradient coupling properties, either fully delocalized or localized. The recent results showing reversible regulation or gating of which pattern is expressed (16) greatly strengthens this hypothesis.

It is interesting that the lag in the high salt-treated case is shorter than the 19–22-flash lag observed in the low salt-treated case where the sequestered domain was depleted by the “CCCP, then BS A” treatment (Table II, lines 4 and 6). One might think that opening the lumen to equilibration with protons otherwise constrained to localized domains should increase the ATP onset lag (as it does when using valinomycin and K$^+$ to collapse the $\Delta \psi^*$ component (see Fig. 1, Ref. 15)). However, the data are consistent with the dual pathways hypothesis by the following reasoning. In the high salt case, if the lumen protons have direct (and first) access to the CF$_{\text{O}}$ under the driving force of the $\Delta \psi^*$, then empty or full buffering domains could be irrelevant because the $\Delta \psi^*$ forces the H$^+$ nearest the CF$_{\text{O}}$ out through the channel. In the low salt case, we speculate that if the CF$_{\text{P}}$ channel is gated shut to the lumen, but open to the domains (cf. Ref. 16 for more details on the gating action), and if in the domain-proton-depleted condition there are fewer protons close to the CF$_{\text{O}}$ channel, then additional flashes could be understood as necessary to fill the domains sufficiently to allow enough protons access to the CF$_{\text{O}}$ channel to accomplish the energization. Moreover, if the localized domains are more in the nature of a hydrogen-bonded chain network for proton relay (35) than an aqueous pore, then it may be expected that few free protons would be situated close to the CF$_{\text{O}}$, especially in the deprotonated amine situation. This is, of course, speculative, but it seems a plausible possibility around which to design other experiments.

Acknowledgments—We thank Drs. J. W. Farquhar, W. A. Beard, and F. C. T. Allnutt for discussions of this work and Janet Hollister for excellent help preparing the manuscript.

REFERENCES
1. Baker, G. B., Bhattacharyya, D., and Dilley, R. A. (1981) Biochemistry 20, 2307–2315
2. Laszlo, J. A., Baker, G. M., and Dilley, R. A. (1984) Biochim. Biophys. Acta 764, 160–169
3. Laszlo, J. A., Baker, G. M., and Dilley, R. A. (1984) J. Bioenerg. Biomembr. 16, 37–51
4. Thog, S. M., and Homann, P. H. (1982) Biochim. Biophys. Acta 670, 221–234
5. Thog, S. M., Johnson, J. D., and Homann, P. H. (1982) FEBS Lett. 145, 259–264
6. Pfister, V. R., and Homann, P. H. (1986) Arch. Biochem. Biophys. 246, 525–530
7. Thog, S. M., and Junge, W. (1983) Biochim. Biophys. Acta 723, 294–307
8. Polle, A., and Junge, W. (1986) FEBS Lett. 198, 263–267
9. Thog, S. M., Belanger, K. M., and Dilley, R. A. (1987) J. Bioenerg. Biomembr. 19, 53–58
10. Dilley, R. A., and Schreiber, U. (1984) J. Bioenerg. Biomembr. 16, 173–193
11. Baker, G. M., Bhattacharyya, D., and Dilley, R. A. (1985) J. Bioenerg. Biomembr. 14, 249–264
12. Beard, W. A., Chiang, G., and Dilley, R. A. (1988) J. Bioenerg. Biomembr. 20, 107–126
13. Ferguson, S. J. (1985) Biochim. Biophys. Acta 811, 47–95
14. Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction, p. 90, Hall Graphics, Plymouth, Great Britain
15. Beard, W. A., and Dilley, R. A. (1986) FEBS Lett. 201, 57–62
16. Chiang, G., and Dilley, R. A. (1987) Biochemistry 26, 4911–4916
17. Beard, W. A., and Dilley, R. A. (1988) J. Bioenerg. Biomembr. 20, 85–106
18. Ort, D. R., and Dilley, R. A. (1970) Biochim. Biophys. Acta 449, 95–107
19. Cramer, T., Flores, S., and Ort, D. R. (1981) in Energy Coupling in Photosynthesis (Selman, B. R., and Selman-Reiner, S., eds) pp. 25–34, Elsevier/North-Holland, New York
20. Witt, H. T., Schledder, E., and Graber, P. (1976) FEBS Lett. 69, 272–276
21. Schledder, E., and Witt, H. T. (1980) FEBS Lett. 112, 105–113
22. Uribe, E. G., and Li, B. Y. (1973) Bioenergetics 4, 435–444
23. Schultz, D., Rottenberg, H., and Avron, M. (1973) Eur. J. Biochem. 39, 453–462
24. Hangarter, R. P., and Good, N. R. (1982) Biochim. Biophys. Acta 681, 397–404
25. Hangarter, R. P., and Dilley, R. A. (1988) J. Bioenerg. Biomembr. 20, 129–154
26. Hangarter, R. P., and Ort, D. R. (1986) Eur. J. Biochem. 158, 7–12
27. Junge, W., Hong, Y. Q., Qiao, L. P., and Viale, A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3078–3082
28. Shavit, N., and Strotmann, H. (1980) Methods Enzymol. 69, 321–324
29. Graber, P., Junesch, U., and Schatz, G. H. (1984) Ber. Bunsen-ges. Phys. Chem. 86, 599–608
30. Abbott, M. S., and Dilley, R. A. (1983) Arch. Biochem. Biophys. 222, 95–104
31. Dilley, R. A., Thog, S. M., and Beard, W. A. (1987) Ann. Rev. Plant Physiol. 38, 348–368
32. Cramer, T. (1982) Curr. Topics Membr. Transp. 16, 215–245
33. Graan, T., and Ort, D. R. (1981) Biochim. Biophys. Acta 637, 447–456
34. Wartesl, G., Golstein, L., and Avron, M. (1974) Eur. J. Biochem. 47, 403–407
35. Nagle, J., and Trisram-Nagle, S. (1983) J. Membr. Biol. 74, 1–14