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Correlation Between Membrane-Localized Protons and Flash-Driven ATP Formation in Chloroplast Thylakoids

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Abstract

Flash-driven ATP formation by spinach chloroplast thylakoids, using the luciferin luminescence assay to detect ATP formed in single turnover flashes, was studied under conditions where a membrane protein amine buffering pool was either protonated or deprotonated before the beginning of the flash trains. The flash number for the onset of ATP formation was delayed by about 10 flashes (from 15 to about 25) when the amine pool was deprotonated as compared to the protonated state. The delay was substantially reversed again by reprotonating the pool upon application of 20–30 single-turnover flashes and 8 min of dark before addition of ADP, P_i, and the luciferin system. In the case of deprotonation by desaspidin, the uncoupler was removed by binding to BSA before the reprotonating flashes were given. Reprotonation was carried out before addition of ADP and P_i, to avoid a possible interference by the ATP-ase, which can energize the system by pumping protons. The reprotonated state, as indicated by an onset lag of about 15 flashes rather than 25 for the deprotonated state, was stable in the dark over extended dark times. The number of protons released by 10 flashes is approximately 30 nmol H^+ (mg chl)$^{-1}$, an amount similar to the size of the reversibly protonated amine group buffering pool. The data are consistent with the hypothesis that the amine buffering groups must be in the protonated state before any protons proceed to the coupling complex and energize ATP formation. Other work has suggested that the amine buffering pool is sequestered within membrane proteins rather than being exposed directly to the inner aqueous bulk phase. Therefore, it is possible that the sequestered amine group array may provide localized association–dissociation sites for proton movement to the coupling complex.

Key Words: Photophosphorylation; proton gradients; chloroplast membranes; proton binding domains.

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Introduction

The protonmotive force developed across (Mitchell, 1966) or within (Williams, 1962) chloroplast, mitochondria, or bacterial membranes is generally believed to drive ATP formation (Boyer *et al.*, 1977). Whether the primary mechanism of proton processing involves localized domains within, or on, the membrane or obligatorily transmembrane, delocalized gradients is as yet unresolved. Studies with chloroplast thylakoids using two very different approaches have supported a localized proton processing hypothesis. One approach measured the onset of ATP formation in short illumination times as influenced by various factors such as the presence of added permeable buffers (Ort and Dilley, 1976; Ort *et al.*, 1976, Graan *et al.*, 1981, Graan and Ort, 1981, 1982, 1983). The other approach utilized chemical modification reagents as probes for membrane–proton interactions (Giaquinta *et al.*, 1974, 1975; Prochaska and Dilley, 1978; Baker *et al.*, 1981, 1982; Tandy *et al.*, 1982; Laszlo *et al.*, 1984a).

This report will bring together certain aspects of the two approaches for the purpose of testing further the hypothesis that localized proton binding domains may be involved in proton movement into the energy coupling complex. The chemical modification experiments have allowed the identification of an array of amine buffering groups having a pK_a near 7.8, which are sequestered within localized domains associated with membrane proteins (Laszlo *et al.*, 1984a). The objective of the experiments reported here was to test whether the length of the lag in onset of ATP formation was influenced by the extent of protonation of the amine buffering array.

Methods[3]

Chloroplasts

Spinach (*Spinacea oleracea*) thylakoids were isolated from greenhouse-grown plants by the method of Ort and Izawa (1973). Chlorophyll assay was by the technique of Arnon (1949).

ATP Assay

ATP formation was measured by the luciferin–luciferase luminescence detection method, as described by Schreiber and del Valle-Tascon (1983). Single-turnover excitation flashes were provided at a 1 Hz frequency. The

[3]Abbreviations: Cl-CCP, *m*-chlorocarbonylcyanide phenylhydrazone; BSA, bovine serum albumin; Ac_2O, acetic anhydride; NGG, *N*-glycylglycine.

reaction cuvette of 10 ml volume was stirred and maintained at 10°C. Addition of reagents could be made during a run through a syringe needle port. Stringently dark conditions were maintained in the room and throughout the storage, transfer, and incubation periods. A low-intensity green safe light located to one side of the working space provided enough light to load the cuvette.

The reaction conditions and the order of addition of reagents, a critical point in these experiments, are specified in the figure legends. The basic reaction medium was, unless specified, 50 mM sorbitol, 50 mM Tricine–KOH, pH 8.5, 0.1 mM methylviologen, and 3 mM $MgCl_2$. Chloroplasts equivalent to 15 μg chl ml^{-1} were added under dark conditions, having been kept in the dark while on ice.

It was necessary to minimize ATP hydrolysis, in the dark, contributing to the formation of proton gradients, so addition of sulfhydryl reagents such as dithiothritol was avoided. Also, the ADP was purified, free of contaminating ATP by the use of a Dowex 1 resin (Cl^- form, 200–400 mesh), according to Shavit and Strotmann (1980). The ADP fractions were pooled, adjusted to pH 6.8 with KOH, and the concentration measured by 260 nm absorbance. To avoid formation of ATP from ADP by the endogenous thylakoid adenylate kinase (Karu and Moudrianakis, 1969), 5 μM diadenosine pentaphosphate was added to the reaction mixtures. The presence or absence of ATPase activity can be readily detected in the assay (Schreiber and Del Valle-Tascon, 1983).

The luciferin–luciferase ATP-dependent luminescence was detected using the LKB ATP monitoring reagent. The contents of one vial were dissolved in 1.5 ml of cold distilled water, and 0.3 ml aliquots were frozen for later use. To each 10-ml reaction mixture was added 100 μl of the luciferin–luciferase suspension followed by 5 μM diadenosine pentaphosphate, 0.1 mM ADP, and 1 mM K_2HPO_4.

Treatments such as a thermal transition or a preillumination were given prior to the addition of the luciferin-luciferase, diadenosine pentaphosphate, ADP, and P_i.

Electron Transfer Assay

Electron transfer activity was measured as before (Baker *et al.*, 1981) using an oxygen electrode, with the basic reaction medium specified above, containing methylviologen as the cofactor. The electron transport activity was used to monitor the response of the thylakoids, used in this study, to the combined effects of low concentrations of uncouplers and acetic anhydride. As explained in Baker *et al.* (1981), this provides an assay for the presence or absence of a membrane-localized pool of amine-buffered protons.

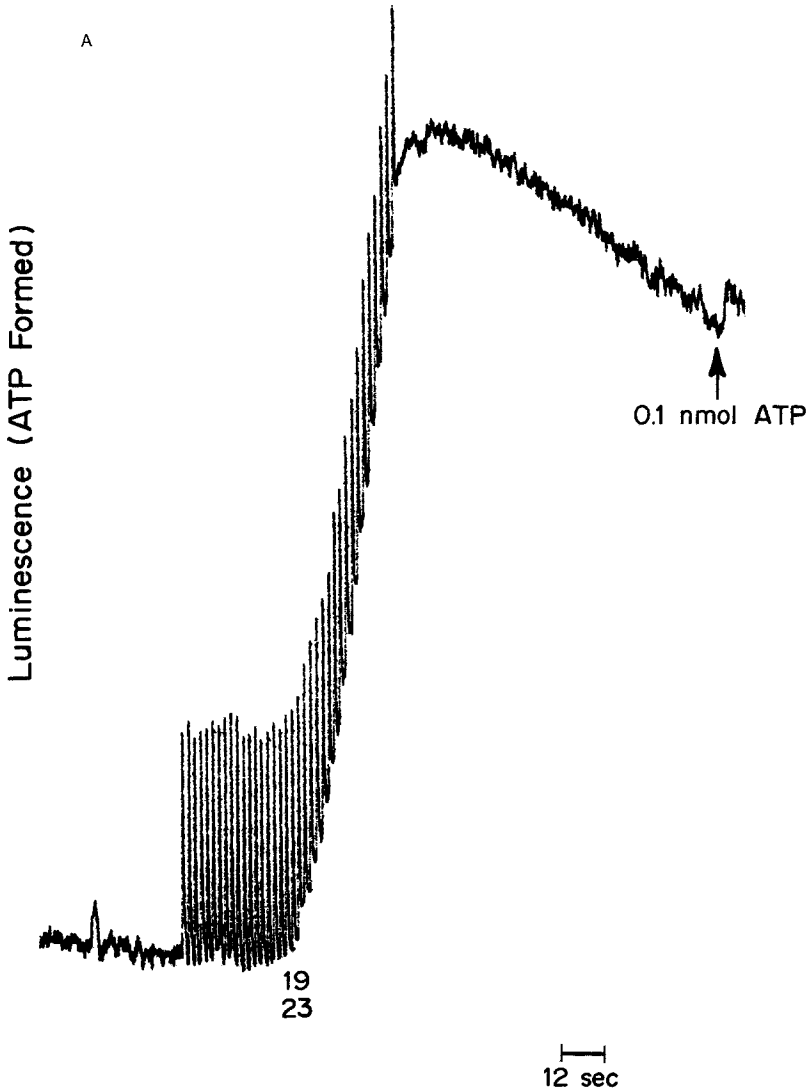

Fig. 1. Typical flash-driven ATP formation assayed by luminescence from the luciferin–luciferase system. (A) The reaction mixture contained, in addition to basic reaction media (cf. Methods), 100 μl LKB luciferin–luciferase reagent, 5 μM diadenosine pentaphosphate, 1 mM P_i, and 0.1 mM ADP. The vertical spike, due to an intentional light leak from the flash, allows detection of the flash event. The ATP formation is evident from the rise of the signal with successive flashes after a 19-flash lag. The addition of 10 μl of 10^{-5} M standard ATP gave a calibration of the ATP yield. (B) The reaction mixture was similar to that for (A), with 20 nM valinomycin added. The sample was preilluminated 3 sec with saturating intensity of white light, followed by 8 min of darkness, after which time ADP, P_i, diadenosine pentaphosphate, and the luciferin components were added.

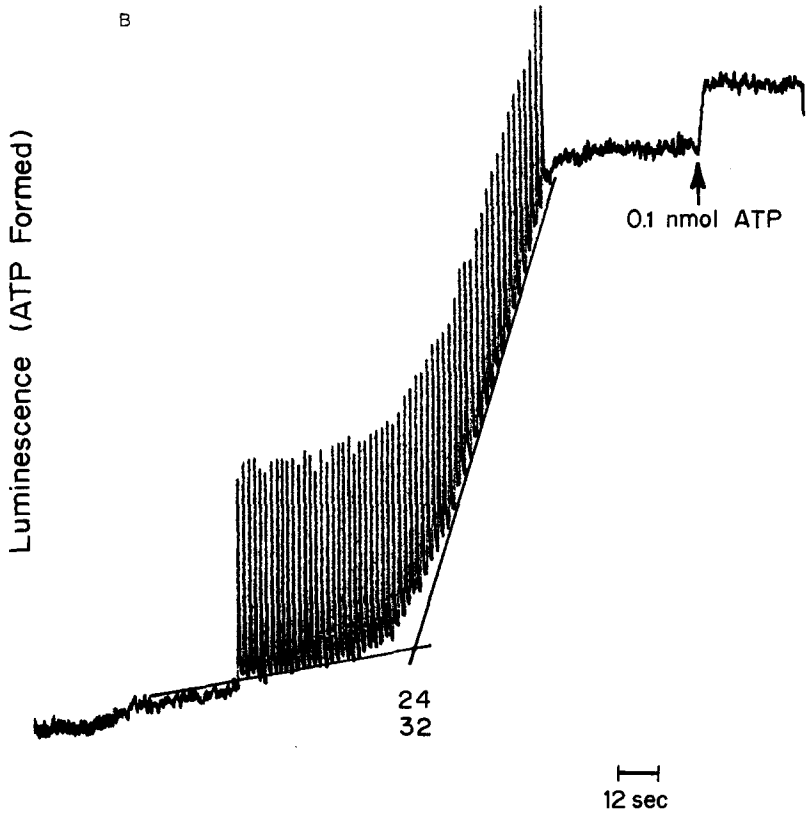

Fig. 1. Continued.

Results

Flash-Driven ATP Formation

A complete reaction mixture in the 10 ml, stirred cuvette contained chloroplasts and the appropriate reaction medium including the luciferin–luciferase. Upon illumination of a dark-adapted sample with a train of single-turnover flashes at 1 Hz frequency, typical responses were as shown in Fig. 1. The transient spikes result from a small leakage of actinic flash light through the filter set, which was allowed as a convenient indicator of beginning and end of flash illumination. Figure 1A shows the result in the absence of valinomycin and Fig. 1B in the presence of 20 nM valinomycin. Two effects of valinomycin are evident: an extension of the lag in the onset of ATP formation and a decrease in the yield of ATP per flash, both of which have been previously reported (Ort and Dilley, 1976; Graan and Ort, 1981;

Hope *et al.*, 1982). The sample used for Fig. 1A showed a significant ATP hydrolysis rate after the flash train, even though no dithiothritol was in the reaction mixture. The ATPase activity was probably due to the fact that the chloroplasts had been freshly isolated from leaves exposed to light in the greenhouse (cf. Galmiche and Girault, 1982). The chloroplasts used for Fig. 1B were isolated from leaves kept in the dark overnight at 2–4°C. The slight

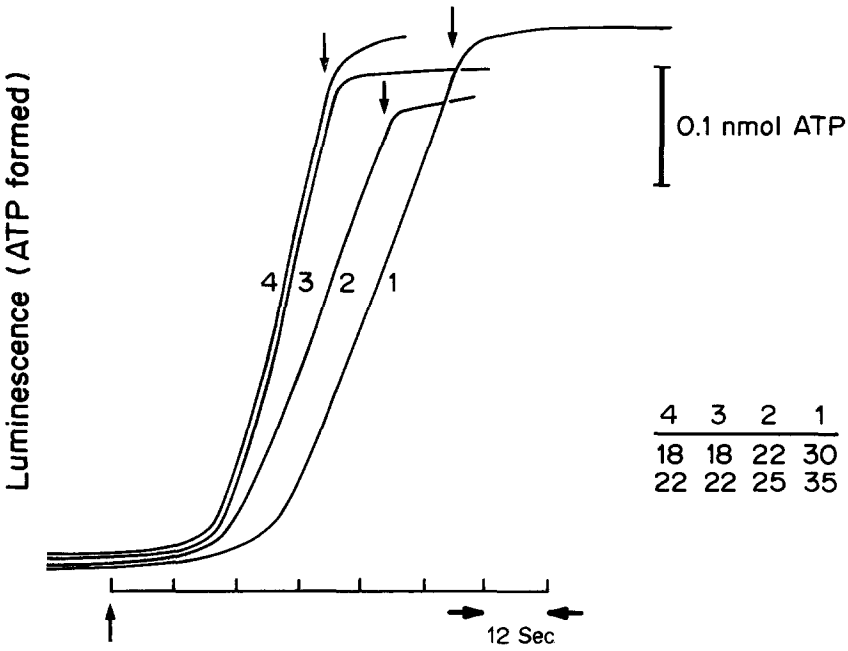

Fig. 2. Desaspidin extends, and preillumination shortens, the lag in onset of ATP formation. Chloroplasts were suspended as in Fig. 1B, except that after the appropriate dark or preillumination treatments, luciferin–luciferase, diadenosine pentaphosphate, ADP, P_i, and 10^{-7} M valinomycin were added. The start of the 1-Hz flash sequences is indicated by the upward pointed arrow, and the end by the downward arrows. A fresh sample was prepared for each treatment, except for curve 4, which is a second cycle. Treatments: *Curve 1.* 10^{-7} M desaspidin was added to the basic mixture, 1 min later 2.5 mg BSA was added to bind the desaspidin; 5 min additional dark incubation followed. During the last minute of darkness the other components of the assay, mentioned above, were added. Note the absence of ATP hydrolysis before and after the flash regime. *Curve 2.* The sample was prepared similarly to that for curve 1. Desaspidin was added, followed by BSA. Two minutes later a 3-sec illumination with saturating intensity of white light was given, followed by 8 min of darkness. The remaining assay components were added, and the flash regime was given. *Curve 3.* Control: BSA was added before the 10^{-7} M disaspidin. After a 3 min incubation in darkness, the flash regime was given. Another control (not shown) was prepared identically but given a 9.5-min dark incubation time prior to the flashes. The ATP onset parameters for that control were essentially identical to those for curve 3 (17 and 23 flashes, respectively, for the two parameters). *Curve 4* is a second flash cycle given to sample 3 after a 4-min dark period following the first flash sequence. The calibration, by addition of 10 μl of 10^{-5} M ATP to each sample, gave a similar value for all the samples.

upward slope in the Fig 1B luminescence trace before the flashes began was probably caused by a small activity of the chloroplast adenylate kinase (Karu and Moudrianakis, 1969; Moudrianakis and Tiefert, 1976).

To simplify the data presentation for the flash-induced ATP formation described below, we drew a continuous line connecting the bottom of the trace of spikes due to the light pulses (see Fig 2, for example). This envelope of the onset kinetics of ATP formation is sufficient to depict the effects observed. The important parameter for these experiments was the number of flashes needed to produce the onset of ATP formation. Two criteria were used to assess this point: (a) the first detectable rise in luminescence (in Fig. 1B occurring at flash number 24 ± 1) and (b) the back extrapolation of the steady rise in the flash-induced fluorescence increase to the x-axis, (in Fig. 1B at flash number 32 ± 1). In subsequent figures these two criteria will be listed on the figure below the trace, as for example, $\frac{24}{32}$. Taken together, the two criteria permit a comparison of the effect of a given treatment on the lag in onset of ATP formation. The ATP yield per flash was calibrated by addition of standard ATP.

It is apparent from Fig. 1B that there was no ATPase activity detected before the flash train. It has been pointed out by Galmiche and Girault (1982) and by Schreiber and Del Valle-Tascon (1983) that ATPase activity occurring in the sample prior to the beginning of the illumination by single-turnover flashes can greatly influence the observed onset behavior and yield per flash of ATP formation. For the experiments of this study we minimized the ATPase activity by: (1) preparing chloroplasts from leaves kept overnight in darkness; (2) avoiding the use of sulfhydryl reagents such as dithiothritol; (3) maintaining low levels of ATP by using purified ADP (see Methods) and by inhibiting adenylate kinase activity with diadenosine pentaphosphate; and (4) by generally using only a single train of flashes with the complete set of assay components present. Certain experiments required a preillumination treatment, but that was given before addition of ADP and P_i. When the above precautions were taken, such treatments did not lead to detectable ATPase activity prior to, or after, the train of flashes used to drive ATP formation (see Fig. 1B, for example).

Rationale for Testing for a Correlation between Lags in Onset of ATP Formation with Proton Buffering Pools

Using acetic anhydride as a chemical modification probe, we have shown that thylakoid membranes contain an array of reactive protein groups, probably amines, having the following properties.

1. Thylakoids as normally isolated and kept dark at ice bath temperatures have an array of about 30 nmol (mg chl)$^{-1}$ of $-NH_3^+$ groups that are metastable in the protonated form. By metastable we mean that, even though

the apparent pK_a of the amine array is near 7.8 (Laszlo *et al.*, 1983), thylakoids suspended in pH 8.6 buffer retain the charged (protonated) form of the amine for extended times. Addition of low concentrations of uncouplers or a brief thermal treatment causes the loss of about 30 nmol H^+ (mg chl)$^{-1}$ from the membrane with a concomitant appearance of an equivalent amount of acetic anhydride-reactive groups (Baker *et al.*, 1981, 1982; Theg *et al.*, 1982; Johnson *et al.*, 1983).

2. In the deprotonated state, the additional reaction with acetic anhydride leads to inhibition of water oxidation (Baker *et al.*, 1981). Three polypeptides believed to be closely associated with the PS II water oxidation reaction (Yamamoto *et al.*, 1983) having M_r (SDS-PAGE) values of 33, 24, and 17, each containing nearly 10% lysine, show large acetic anhydride labeling change patterns (Laszlo *et al.*, 1984b). Proton accumulation linked either to electron transfer (Baker *et al.*, 1981) or ATP hydrolysis (Baker *et al.*, 1982) can reverse the anhydride sensitivity of electron transport and the labeling level, in keeping with the concept that the amine group array is in acid–base equilibrium with the translocated protons.

The 8 KD CF_0 proton channel protein also shows anhydride labeling (at the lysine 48 residue) that is variable depending on the presence of uncoupler and light-induced proton accumulation (Tandy *et al.*, 1982).

3. The array of amine groups sensitive to acetic anhydride is buried in sequestered regions of membrane proteins, so as not to be in ready equilibrium with the inner aqueous space (Laszlo *et al.*, 1984a). The amine array titrates with an apparent pK_a near 7.8, when an uncoupler was used to allow rapid equilibration of the amine group array with the external phase.

The properties of the sequestered amine group array lead us to the hypothesis that the special pool of amines may be involved in proton processing into the ATP formation apparatus. Moreover, these properties provide a means of testing the hypothesis, by studying the onset of ATP formation in single-turnover flashes.

The experiment is visualized as follows: the thylakoids will be poised in conditions where the amine group array is either protonated or unprotonated. The onset of ATP formation in a series of single-turnover light flashes will be measured in each state. Those data will be compared to a situation where the thylakoids are first deprotonated in the dark, and then given a light regime that is known to reprotonate the amine groups, followed by a second flash sequence with ADP + P_i present so as to measure the onset in ATP formation.

The deprotonation treatment could be either a thermal treatment or addition of uncoupler (Baker *et al.*, 1982). For subsequent phosphorylation measurements the uncoupler must be removed, and that was accomplished, in the cases of desaspidin and Cl-CCP, by adding bovine serum albumin

(Schreiber, to be submitted). Apparently, Cl-CCP or desaspidin bind strongly enough to added BSA that with concentrations of either uncoupler in the range of 0.1–0.5 μM, with the thylakoid suspension at 20 μg ml^{-1} chlorophyll, the uncouplers will partition away from the thylakoids onto the BSA. This was first observed in luminescence experiments (Schreiber, unpublished results).

Effect of Reversible Uncoupler Treatment on Lags in Onset of ATP Formation

Desaspidin, pH 8.5. Figure 2 shows that 0.1 μM desaspidin given for 1 min in the dark followed by 2.5 mg BSA to remove the desaspidin from the membrane results in an extension of the onset of ATP formation from 18 flashes (curve 3, control, BSA added before the desaspidin) out to 30 flashes (curve 1).

A separate sample (curve 2) was treated with desaspidin followed by BSA, but then 3 sec of white light was given (methylviologen was present, but not ADP or P_i), followed by 8 min of dark to allow complete relaxation of the transmembrane proton gradient. At 7 min dark time, luciferin–luciferase, diadenosine pentaphosphate, ADP, and P_i were added and the ATP formation was elicited by the flashing-light regime. Curve 2, Fig. 2 shows that the onset lag for this treatment was shortened to 22 flashes, compared to the 30-flash lag for the sample not preilluminated. Curve 4 shows a control consisting of a second flash cycle, after 4 min dark, given to the sample used for curve 3. This suggests that a preillumination did not cause a shorter lag in general, but rather that the lag shortening caused by preillumination of sample 2 compared to sample 1 was related to the uncoupler effect giving a *longer* lag to begin with. A further control, not shown, was a sample identical to that for curve 3, but with a 9.5 min dark time before starting the flash train. It also gave a relatively short lag similar to that for curve 3 (17 and 23 flashes, respectively, for the two onset criteria discussed above). This establishes that the longer lag found in sample 1 was not due to the 5 min of dark incubation time given to sample 1.

Results similar to those of Fig. 2 were found when 36 single-turnover flashes rather than 3 sec of continuous light were given for the preillumination treatment (data not shown). In an experiment of that type, using a different chloroplast preparation and 0.2 μM desaspidin, the ATP formation onset lag parameters were $\frac{38}{48}$, $\frac{29}{35}$, $\frac{23}{28}$, flashes for treatments corresponding to curves 1–3, respectively, of Fig. 2. The effect of the preillumination was to shorten the lag by about 10 flashes, consistent with the results of Fig. 2.

Another control, not shown, consisted of giving a second cycle of flashes to a sample similar to that used for curve 1, to check whether the longer lag observed for sample 1 was a permanent condition induced by that particular

treatment. The second cycle showed a lag shorter than the first cycle by about 10 flashes (see curves 1 and 1′ of Fig. 4, for a similar experiment).

Figure 3 shows that 0.2 μM desaspidin inhibits flash-driven ATP formation about 70% while extending the lag in onset from $\frac{22}{26}$ to $\frac{32}{35}$ flashes (in comparing curves 1 and 2, note that the same sample was used). Addition of BSA to that sample, after the second flash cycle, resulted in shortening the lag parameters back to $\frac{23}{27}$ flashes. The yield of ATP for that flash sequence was not restored to the control level, an effect also seen in the data of Fig. 2. Although not shown, there was a detectable ATPase activity after the second cycle of flashes.

Cl-CCP, pH 8.5. Another uncoupler, Cl-CCP at 0.3 μM, was tested in a phosphorylation experiment similar to that with desaspidin, and the results agreed well. Valinomycin was not used due to its synergistic action with Cl-CCP, the pair being a very potent uncoupler of phosphorylation (Karlish *et*

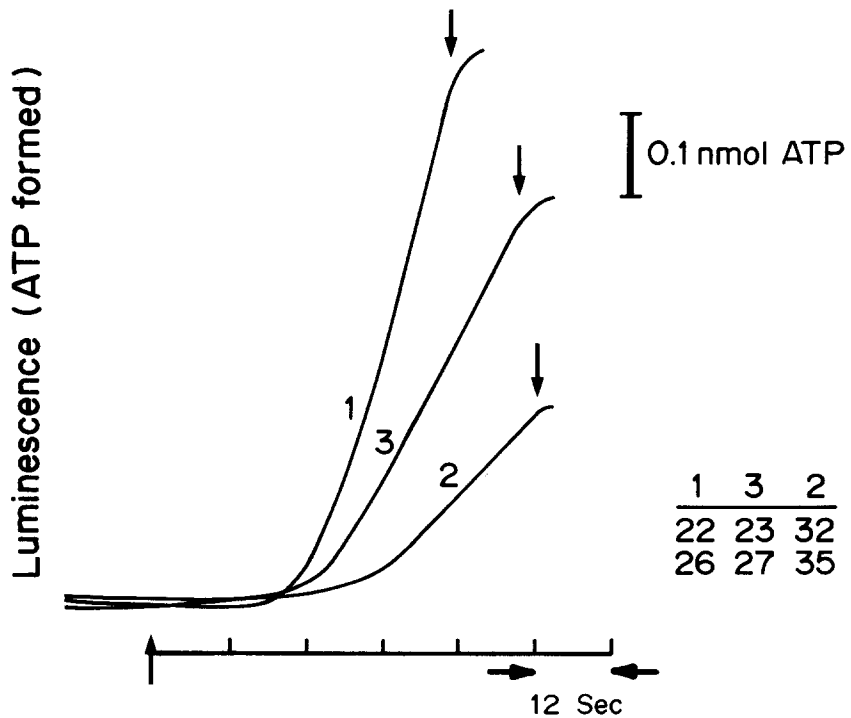

Fig. 3. Effect of desaspidin on flash-driven ATP formation and reversal of the effect by BSA. A single sample was used for this experiment. The sample was prepared similarly to that for Fig. 1A but with 50 nM valinomycin. *Curve 1.* The sample was given the first flash sequence, followed by 3 min darkness. *Curve 2.* Desaspidin (0.2 μM) was added and the next flash series given, followed by 2 min darkness during which 2.5 mg BSA was added. *Curve 3.* The flash sequence was given after the addition of BSA.

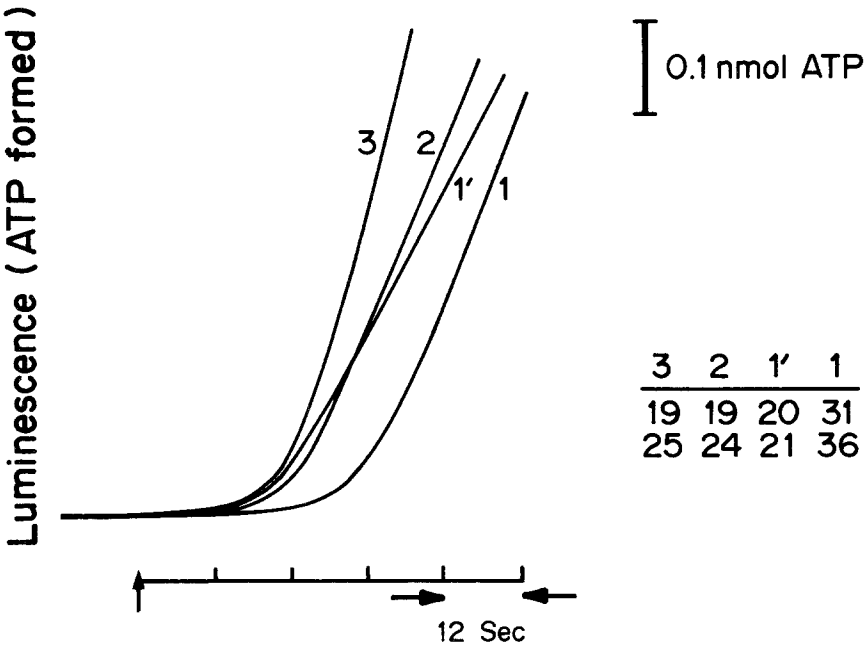

Fig. 4. Cl-CCP and BSA effects on the lag in onset of ATP formation. The experiment is similar in plan to that of Fig. 2, except that no valinomycin was used. *Curve 1.* Chloroplasts in the basic mixture were given 0.3 μM Cl-CCP, then after 1 min darkness, 2.5 mg BSA was added. After an additional 5 min dark incubation, the remaining components for the ATP assay were added and the flash regime started. *Curve 1'.* Same sample as for curve 1, but the second cycle of flashes following a 5-min dark period after the first flash sequence. *Curve 2.* Similar to curve 1, but with 36 single-turnover flashes given before adding ADP, P_i, and the remaining assay components. Five minutes dark time elapsed after the preillumination flashes. *Curve 3.* Control: BSA was added before the 0.3 μM Cl-CCP.

al., 1969). Figure 4 shows that 0.3 μM Cl-CCP for a 1 min dark treatment, followed by BSA to remove the uncoupler from the thylakoids, caused about a 12-flash extension of the lag in onset of ATP formation (compare curve 1 with curve 3). A similar sample was treated with the uncoupler, followed by BSA, and then was given 36 single-turnover flashes after the BSA addition, but before addition of ADP, P_i, luciferin–luciferase, and diadenosine pentaphosphate (curve 2). The results indicate that the preillumination flashes caused the phosphorylation onset lag to be shortened back to the control value (compare curve 2 with curve 3).

Curve 1′ is a second cycle of flashes given to sample 1 after a 5-min dark period, showing a return to a more rapid onset in ATP formation. This control shows that there was nothing permanently impressed on sample 1 that caused the first cycle to show the longer lag.

Cl-CCP at 0.3 μM caused the ATP yield to be inhibited about 32%, when no BSA was added to bind the uncoupler (data not shown).

Desaspidin and Cl-CCP, pH 7.0. As will be discussed in detail below, the data of Figs. 2 and 3 are consistent with the hypothesis that the presence or absence of the pool of amine-buffered protons can significantly influence the onset lag of ATP formation. Another way to test this is to do the same type of experiment as that of Fig. 2, but at pH 7.0, which is nearly 1 pH unit below the pK_a of the sequestered amine group array. If the above rationale is correct, at pH 7.0, there should be no extension of the lag due to addition of uncoupler, because the lower external pH should provide sufficient protons to keep the amine groups in the $-NH_3^+$ state. Figure 5 shows that that was indeed the case. With desaspidin added either before the BSA or after, the lag in onset of ATP formation was identical. Thirty-six single-turnover flashes given to one of the samples did not decrease the lag length.

An experiment with Cl-CCP at pH 7.0 similar to the pH 8.5 experiment of Fig. 4 indicated that at the lower pH, the Cl-CCP did not cause an extension of the lag, in agreement with the pH 7.0 desaspidin result of Fig. 5 (data not shown). In the Cl-CCP pH 7.0, experiment, the lag length for all

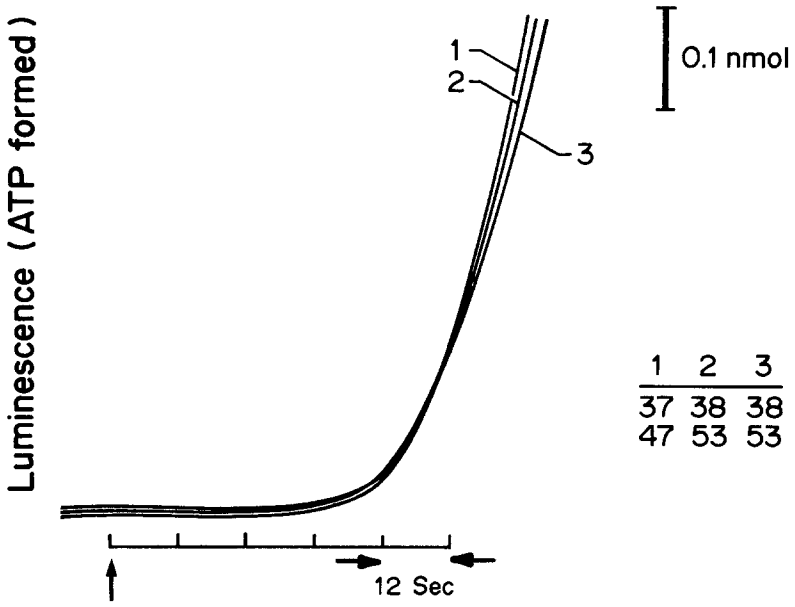

Fig. 5. Effects of desaspidin and BSA at pH 7.0. The experiment was similar to that of Fig. 2 except the pH was adjusted to 7.0 *Curve 1.* Similar in plan to curve 1 of Fig. 2, but desaspidin was given at 0.2 μM, then 2.5 mg BSA was added, followed by 6 min of dark prior to the flash sequence to drive ATP formation. *Curve 2.* Similar to curve 1, but 2 min after 2.5 mg BSA addition, 36 single-turnover flashes were given, followed by 6 min darkness. The remaining components of the ATP assay were added during the last minute of darkness, prior to the ATP formation assay. *Curve 3.* A control with 2.5 mg BSA addition before the desaspidin, followed by 2 min of darkness, addition of the remaining components for the ATP assay, then the flash sequence.

treatments was similar to the lag lengths for the pH 8.5 control sample which had BSA added before the Cl-CCP (data of Fig. 4).

Thermal Treatment

The other approach used in the past work to deplete the metastable proton pool was a brief thermal treatment (Baker *et al.*, 1981, 1982). It was also used in these experiments, with the results shown in Fig. 6. The water circulation jacket around the 10-ml reaction cuvette was connected via a

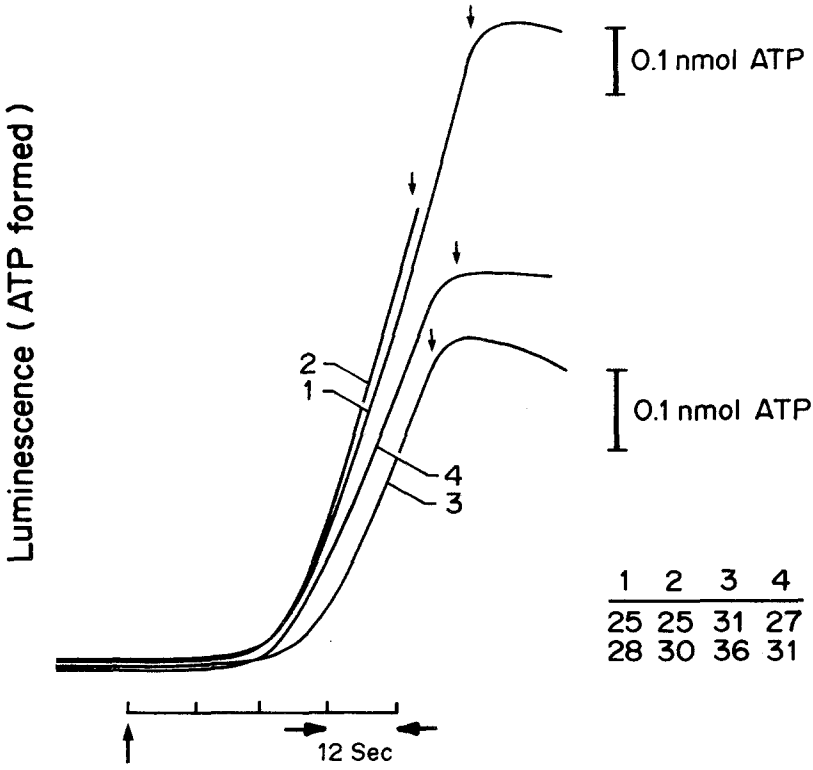

Fig. 6. Thermal treatment and preillumination effects on the lag in ATP formation onset time. The experimental conditions were as in Fig. 2, with 20 nM valinomycin present in all samples. The thermal treatment was given as described in Methods, with the high temperature bath set at 33°C. *Curve 1.* Control sample, no preillumination. *Curve 2.* Control sample, preillumination of the thylakoid suspension with 24 flashes (1 Hz). After a 4-min wait in darkness, ADP, P_i, diadenosine pentaphosphate, and the luciferin–luciferase were added as in Fig. 2, followed by the flash train. *Curve 3.* Thermally treated sample, 33°C for 1 min, returned to 10°C and kept 4 min in dark. The components listed above for curve 2 were added in the dark and then the flash series was given. *Curve 4.* Thermally treated as for curve 3, but after 1 min following return to 10°C, 24 flashes (1 Hz) were given. After 4 min in dark, the rest of the assay components were added and the flash train was started.

switching valve to either a 33°C or a 10°C bath. The thermal treatment of the thylakoid suspension for those experiments consisted of switching the valve to the 33°C bath for 1 min, then back to the 10°C bath. After 1 min at 10°C the 24-flash preillumination or dark treatments were given, a 4-min dark period followed during which the reagents needed for the ATP assay were added, and then the flash sequence was given to drive ATP formation. The controls for this experiment were samples that did not receive a thermal treatment. Figure 6 shows that the thermal treatment delayed the onset parameters of ATP formation from $\frac{25}{28}$ flashes to $\frac{31}{36}$ flashes. The preillumination, 24-flash treatment given after thermal transition shortened the lag to $\frac{27}{31}$ flashes. The controls—not thermally treated—showed no shortening of the onset lag due to the preillumination treatment (compare curves 1 and 2).

Desaspidin Effects on Acetic Anhydride Inhibition of Electron Transfer

Desaspidin was tested for its comparative effectiveness in producing the proton leak conditions previously reported with FCCP, nigericin, and gramicidin (Baker *et al.*, 1981). The assay used was the uncoupler-induced inhibition of electron transport by acetic anhydride addition in the dark and protection against inhibition when anhydride was added in the light (Baker *et al.*, 1981). Desaspidin gave a response similar to, but less potent than, nigericin (Table I). Under illuminated conditions, with either uncoupler present before addition of 3 mM acetic anhydride, there was no inhibition. Nigericin at 0.5 μM under dark conditions led to a 79% inhibition by 3 mM acetic anhydride. In darkness with 0.1 or 0.2 μM desaspidin, acetic anhydride addition gave 51 and 54% inhibition, respectively. Addition of BSA to a sample that had been exposed to 0.2 μM desaspidin, followed by 5 sec of illumination, permits the thylakoids to regain the anhydride-resistant state (Part B, Table I). Thus, by the two criteria tested—restoration of ATP formation and restoration of the photosystem II resistance to acetic anhydride inhibition—BSA addition to uncoupler-treated thylakoids causes the uncoupler effect to be nullified.

Discussion

The objective of these experiments was to compare the onset lag length of ATP formation in thylakoids which were treated so as to have an endogenous amine-group buffering array either in the fully protonated or in the deprotonated state. The experimental demonstration of an endogenous buffering array with an apparent pK_a near 7.8 is clear (Baker *et al.*, 1981; Laszlo *et al.*, 1984a; Theg *et al.*, 1982). It is also clear that the buffering array can be experimentally manipulated so as to be poised either in the fully protonated or

Table I. Desaspidin, Bovine Serum Albumin, and Preillumination Effects on Acetic Anhydride Inhibition of Electron Transfer in Thylakoids[a]

Treatment prior to addition of acetic anhydride	Electron transfer rate (μeq (hr mg chl)$^{-1}$	Percent (%) inhibition
A.		
1. Light, 0.5 μM nigericin	921	0
2. Dark, 0.5 μM nigericin	192	79
3. Light, 0.2 μM desaspidin	920	0
4. Dark, 0.1 μM desaspidin	450	51
5. Dark, 0.2 μM desaspidin	428	54
B.		
1. Dark, BSA before desaspidin	207	0
2. Dark, desaspidin before BSA	110	47
3. Desaspidin before BSA, 5-sec light, 4 min dark, then Ac_2O	207	0
4. As above, with 8 min darkness after the BSA	193	7

[a]A. Test for whether desaspidin acts similarly to the other uncouplers used previously. Chloroplast thylakoids were suspended in 50 mM sorbitol, 50 mM Tricine-KOH, pH 8.5, 3 mM $MgCl_2$, 0.1 mM methylviologen, and with 15 μg chl (ml)$^{-1}$. The Clark-type oxygen electrode chamber was shielded from room light and kept at 20°C with rapid stirring. After low levels of nigericin or desaspidin were added, the reaction was either kept for 30 sec in darkness or illuminated with saturating white light (heat-filtered through a copper sulfate solution in water). Acetic anhydride, 3 mM, was then added to the suspensions and after 15 sec of reaction time, 50 mM *N*-glycylglycine was added to quench the unreacted anhydride and the actinic light was turned off (when it was used). Nigericin at 1 μM was added to assure maximum uncoupling and then the actinic light was turned on again for the assay of electron transfer rates. B. Testing if bovine serum albumin added after desaspidin allows preillumination to reverse the desaspidin-induced acetic anhydride sensitivity of electron transport. Chloroplasts (a different batch than that used for part A) were suspended as for part A, but at 10°C, and with 1.0 mg ml^{-1} BSA added as indicated. 1. Chloroplasts were in the dark and BSA was added before the desaspidin. Acetic anhydride (3 mM) was added and, after 15 sec, 50 mM NGG quench. Before turning on the light to assay the effect on electron transfer, 1 μM nigericin was added. 2. Chloroplasts kept in the dark and 0.2 μM desaspidin was added. After 1 min, BSA was added with another 1-min wait, then 3 mM acetic anhydride was added and, after 15 sec, 50 mM NGG quench, then the 1 μM nigericin. 3. Similar to experiment 2 but, 1 min after BSA addition, 5 sec of white light was given, followed by 4 min of darkness. Acetic anhydride, NGG quench, and 1 μM nigericin were then given as in experiment 1. 4. Similar to experiment 3, but 8 min of darkness followed the 5-sec light exposure.

the deprotonated state (Baker *et al.*, 1981; Laszlo *et al.*, 1984a). Now the location of the 30 nmol (mg chl)$^{-1}$ of membrane-protein buffering groups could be anywhere behind the permeability barrier of the thylakoid. The groups could be insulated away from the regions associated with proton production competent to drive ATP formation, or they could be fully exposed to the energy-coupling proton gradients. The present experiments tested for these alternatives, and we found evidence for the latter, more interesting, case.

The pretreatments necessary to manipulate the protonation state of the metastable proton pool were judged adequate, as evidenced by the data of

Table I and Fig. 3. The uncoupler desaspidin, given to dark-held thylakoids, mimics the effects of the previously studied uncouplers FCCP, nigericin, and gramicidin (Baker *et al.*, 1981) in releasing the bound protons allowing the formation of the uncharged, acetic anhydride-sensitive amine functions. Acetylation of those newly formed $-\ddot{N}H_2$ functions results in inhibition of water oxidation activity, a diagnostic indicator of the proton-depleted state. Bovine serum albumin strongly binds desaspidin and can remove it from the thylakoids, allowing the $-NH_3^+$ form of the buffering array to be restored by the redox proton pumps (Part B, Table I). Those results agree with the phosphorylation data of Fig. 3, showing that BSA reverses the inhibitory effect of desaspidin on ATP formation. Similarly, 0.5 mg ml^{-1} BSA essentially completely reversed the inhibitory action of 0.5 μM Cl-CCP against ATP formation (unpublished results, experiments carried out by David Overdier and Steven Theg).

The data of Figs. 2, 4, and 6 clearly show that the treatments which were expected to deplete the metastable pool of membrane-phase buffered protons caused pronounced extensions of the lags in onset of ATP formation. After the proton depletion treatments (using either desaspidin or Cl-CCP, or a thermal treatment), but before adding ADP and P_i, a preillumination regime with a 3-sec light pulse or 24 to 36 single-turnover flashes restored the shorter lags. Interpretation of the results requires that we address three questions: (1) Are the different onset lags due to proton effects, or can the data be equally well explained by phenomena not involving protons? (2) What significance should be ascribed to the magnitude of the effects on the phosphorylation onset lag? (3) If proton interactions are invoked as the explanation, what can be concluded regarding the location of the proton pools, i.e., are localized or delocalized proton gradients to be invoked?

1. Proton effects seem the most reasonable explanation for the data because a proton gradient is believed to be the driving force for ATP formation. Previous studies of chloroplast phosphorylation onset lags, regardless of the details of interpretation, have generally supported the notion that the length of the lag is determined by the buildup of the protonmotive force (Ort *et al.*, 1976; Davenport and McCarty, 1980; Vinkler *et al.*, 1980). Invoking effects on activation of the CF_1 complex due to the treatments also would involve protons, as there is evidence that proton flux through the CF_0–CF_1 unit is the probable cause of activation (Gräber, 1982; Abbott and Dilley, 1983). The latter authors have shown that there is a component of proton efflux that occurs in the light which can be correlated closely with adenine nucleotide exchange, an event believed to be associated with activation of the CF_1 complex.

Further compelling evidence that the lag effects involve protons comes, in the present work, from noting that the treatments which extend (uncoupler

addition) or shorten (proton-linked redox or ATPase activity) the lag have been shown previously to involve proton release and proton accumulation, respectively (Baker *et al.*, 1981, 1982; Ort and Dilley, 1976; Ort *et al.*, 1976, Graan and Ort, 1981).

2. The magnitude of the extension and the shortening of the lags was about ten flashes. Ten flashes should yield about 30 to 35 nmol H^+ (mg chl)$^{-1}$ translocated into the membrane, according to Graan and Ort's (1982) measurement that a single-turnover flash yields about 3.5 nmol H^+ (mg chl)$^{-1}$. Thirty nanomoles H^+ (mg chl)$^{-1}$ corresponds well with the size of the sequestered proton buffering pool determined by the acetic anhydride probe. The close correspondence in these numbers strongly supports the hypothesis that the 30 nmol (mg chl)$^{-1}$ of buffering groups are located so as to directly interact with protons released by the electron transport reactions.

Moreover, the data show that the buffering groups must be in the protonated state before protons build up the required protonmotive force for ATP formation and/or proceed to the CF_0–CF_1 coupling complex. Theg and Junge (1983) have shown a related phenomenon using neutral red as a probe for pH changes associated with thylakoid membrane electron transfer. They found that after gramicidin treatment, the flash-induced neutral red changes attributed to pH changes in the lumen space due to proton release in water oxidation were delayed until seven or eight flashes; i.e., some pool had to be filled with protons before acidification of the lumen space occurred.

The interpretation we have given above is supported by the data of Fig. 5, inasmuch as at pH 7.0 there was no effect of the desaspidin or preillumination light flashes on the length of the onset lag. We know that at pH 7.0 the membrane buffering pool probed by the acetic anhydride, with a pK_a near 7.8, is mostly protonated [not reactive with the anhydride (Laszlo *et al.*, 1984a)]. Therefore, we can explain the lack of effect, at pH 7.0, of desaspidin or preillumination treatments on ATP onset lags as due to the protonated state of the buffering pool being always present.

When a thermal treatment was used to deplete the metastable proton pool (Fig 6), the results showed an extension in lag of only about six flashes, compared to the nondepleted control. In other experiments (Baker, Laszlo, and Dilley, unpublished results), it has been noticed that the thermal treatment renders the membranes somewhat more leaky to protons than untreated membranes. That is, an illumination treatment will restore the thermally depleted buffering pool to the protonated state (e.g., Fig. 1 of Baker *et al.*, 1982); but at an external pH near 8.5, the thylakoids do not retain the metastable proton pool as tightly as do control thylakoids. This could explain why the onset lag parameters of curves 3 and 4 of Fig. 6 are closer together than for comparable preillumination effects when an uncoupler was used to initially dissipate the metastable proton pool (Figs. 2 and 5).

Valinomycin was used in the experiments of Figs. 2 and 3 at 100 and 50 nM, respectively. Although valinomycin plus K^+ extends the phosphorylation onset lag as in Fig 1, and as expected from previous work (Ort and Dilley, 1976), the antibiotic had little or no effect on the *further extension* of the lag due to the proton depletion treatment, nor on the shortening of that longer lag due to a preillumination treatment. In the experiments with Cl-CCP, no valinomycin was used, and the proton depletion treatment extended the onset lag, and refilling the buffering pool shortened it (Fig. 4). Therefore, it may be concluded that the phenomena observed herein are not measurably influenced by the presence or absence of the electrical component of the protonmotive force.

3. Taken alone, the data of this report do not test directly for the bioenergetic involvement of localized compared to delocalized proton gradients. However, two lines of evidence support the hypothesis for a localized proton processing mechanism. (a) Other work has shown that the "special pool" of about 30 nmol (mg chl)$^{-1}$ buffering groups, sensitive to acetic anhydride and reversibly deprotonated and protonated by the treatments used here, do not reside in, nor equilibrate quickly with, either the inner aqueous phase or the external aqueous phase (Laszlo *et al.*, 1984a). (b) The present results suggest that the "special pool" buffering array is located *between* the proton sources—the redox protolytic reactions—and the CF_1 proton sink which utilizes the proton fluxes to drive ATP formation. That is, protons that seem to be required to fill the "special buffering pool"—a localized domain—appear to do so *while* the onset of ATP formation is retarded. The simplest interpretation is that the special pool buffering array must be protonated before protons from the redox reactions can build up the required protonmotive force for ATP formation. Stated another way, the endogenous amine buffering array is not isolated away from the domain(s) available to protons involved in the initial events of energizing ATP formation. This does not necessarily rule out the possibility that the "special pool" of buffering groups is in *series* both with the bulk lumen space and "downstream" from that, the CF_0–CF_1 complex. That is, there could be localized proton processing pathways close to the redox proton sources leading to the lumen space, which then connect to the CF_0–CF_1. However, that model would not be consistent with the findings that: (a) the anhydride-sensitive buffering pool does not readily equilibrate with the lumen bulk phase; (Laszlo *et al.*, 1984a), and (b) the ATPase proton pump is just as effective as the redox proton pumps in reprotonating a depleted buffering pool (Baker *et al.*, 1982). Because the bulk lumen phase is not, on the time scale of many minutes, in equilibrium with the special buffering pool array, it cannot, at the same time, be an obligatory pathway for protons derived from ATPase proton pumping to reach the

buffering array. Points (a) and (b) above are not consistent with such a model. Therefore, the experimental data are more consistent with the initial events in energy coupling (i.e., entire proton processing pathway) being localized. Yet, this leaves unanswered the question of how the lumen bulk phase gets acidified by redox or ATPase proton pumps, which is believed to happen (Portis and McCarty, 1974; Davenport and McCarty, 1981). One explanation offered previously (Dilley *et al.*, 1982) might be that the lumen bulk-phase proton concentration equilibrates relatively slowly with the protons in the CF_0 channel—at least slower than proton movement in the localized domains. More work is needed to clarify this question as well as to understand the energy-coupling role of the sequestered special pool of buffering groups.

The pK_a of the buffering group array probed by these techniques is near 7.8 (Laszlo *et al.*, 1984a). At that pH, it is clear that the sequestered array is mostly in the protonated state *in situ* under dark or light conditions. That is, the array is not a candidate for involvement in energy-storing or transducing reactions associated with protonation–deprotonation cycles. Yet the data of this report implicate the buffering group array in proton processing events in energy coupling. A hypothesis based on the ideas of Nagle and Morowitz (1978) [cf. Dunker and Marvin, (1978) for a different concept concerning how protein structures might be involved as proton pathways] is that the buffering group array may provide proton-hopping sites in a pathway from the proton sources, water oxidation and plastohydroquinone oxidation, to the CF_0–CF_1 proton sink. The "proton wire" concept remains hypothetical and needs experimental testing (Nagle and Tristram-Nagle, 1983) before it can be accepted as a proper model for proton movement through membranes. The present results must also be subjected to more critical testing, but there is an obvious and tantalizing connection between the present results and the proton wire concept.

It remains to be learned which membrane proteins carry the amine functions that constitute the special array of buffering groups and how they are arranged in the thylakoid membrane. The 8 KD CF_0 proton channel protein has one of the lysine amine groups probed by the acetic anhydride technique (Tandy *et al.*, 1982). Three proteins associated with the water-oxidizing system (Yamamoto *et al.*, 1983; Baker, 1983; Laszlo *et al.*, 1984b to be submitted) also show large anhydride reactivity differences between energized and uncoupler-treated states. Some of the directions for future work will be to test if the amine groups of these proteins are buried within the membrane structure or exposed at the membrane interface, as well as to test whether proton hopping along the proteins occurs as suggested by the Nagle–Morowitz hypothesis. It is of interest that the evidence implicates proteins at one of the proton sources, water oxidation, and at the CF_0–CF_1

sink, as showing the properties of a sequestered domain. This is consistent with expectations for, but of course not final proof for, a localized proton-processing domain involved in energy coupling.

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