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Interaction of Photosystem I-Derived Protons with the Water-Splitting Enzyme Complex. Evidence for Localized Domains

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Abstract

The induction of millisecond delayed fluorescence mediated by PS I-dependent proton pumping has been used as an indicator of the time course with which those protons equilibrate with sites on the oxygen-evolving enzyme complex (Bowes, J. M., and Crofts, A. R. (1978). *Z. Naturforsch.* **33C**, 271–275). We found that the induction curves were retarded by a reversible exposure of non-energized thylakoids to low concentrations of the uncoupler, desaspidin, at alkaline, but not at neutral, pH. The induction curves were not retarded by increasing the buffering capacity of the thylakoid lumen with Tricine, and were inhibited by the energy transfer inhibitors, dicyclohexylcarbodiimide (DCCD) and triphenyltin chloride (TPT). These data suggest that (i) the catalytic site of the water-splitting complex is located in proton-sequestering membrane domains, rather than at the lumen-exposed inner membrane surface, (ii) protons released during PS I-mediated electron transport might equilibrate with protonatable sites on the oxygen-evolving complex without passing through the lumen, and (iii) those protons may travel over specific conducting pathways which can be blocked by DCCD and TPT.

Key Words: Photosystem II; membrane domains; delayed fluoresence.

Introduction

During the past ten years evidence has accumulated indicating that thylakoid membranes can hold a population of protons out of equilibrium with those in the outer and inner aqueous bulk phases for times on the order of two hours unless uncouplers are added (Laszlo *et al.*, 1984a). The proton sequestering domains have been suggested to provide localized diffusion pathways for protons to follow between their sites of release from electron transport

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components and the chloroplast coupling factors (Dilley *et al.*, 1981). Initially it was thought that only those protons released during PS II[3] electron transport could gain ready access to the domains (Baker *et al.*, 1981). Later experiments, however, demonstrated that protons liberated by PS I-mediated electron transport, as well as those pumped by the CF_1–CF_0 complex during ATPase activity, could also reprotonate domain-localized buffering groups (Baker, 1983; Dilley, 1986). Recently, it was shown that the domains must be filled with protons before ATP synthesis can be initiated by flashing light (Dilley and Schreiber, 1984; Theg and Dilley, unpublished results). Thus, the existence of the domains is intimately related to the controversy concerning localized vs. delocalized chemiosmotic mechanisms of ATP synthesis in chloroplasts (Ferguson, 1985; Haraux, 1985).

The oxygen-evolving complex (OEC) is widely believed to be located at the inner surface of the thylakoid membrane (Diner and Joliot, 1977; Akerlund and Jansson, 1981; Barber, 1984). Accordingly, it is expected to be in chemical equilibrium with solutes in the lumen (Bowes and Crofts, 1978). Yet, it was demonstrated that protons released by this enzyme complex during the oxidation of water enter the domains first, before, if ever, passing into the lumen (Theg and Junge, 1983; Polle and Junge, 1986). These results suggest that the components of the OEC that participate directly in the protolytic oxidation of water should face the domains, not the lumen. This view was supported by the purported release of Mn into the domains upon denaturation of the OEC (Miller and Cox, 1983), and by the observation that the lower pH of the domains, compared to the external medium, imparted an extra stability of the OEC against Mn extraction that could be eliminated by adding uncouplers to chloroplasts held in the dark (Theg *et al.*, 1982).

In this paper we have examined the question of the location of the OEC using a different, independent technique developed by Crofts and colleagues (Wraight and Crofts, 1971; Bowes and Crofts, 1978). In it, chloroplasts are exposed to a number of flashes to set the OEC in an S-state associated with proton release, then the system is poisoned with DCMU to prevent further S-state advancement. The chloroplasts are then exposed to light in the presence of artificial protolytic electron donors to PS I. The resulting proton pumping activity leads to equilibration of proton binding sites on the S-states

[3] Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; CF_0, hydrophobic protein complex of the chloroplast coupling factor; Chl, chlorophyll; DAD, diaminodurene; DBMIB, 2,5-dibromothymoquinone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,6-dimethylbenzoquinone; DQH_2, durohydroquinone; FeCN, $K_3Fe(CN)_6$; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, oxygen-evolving complex; P680, photosystem II reaction center; PQ, plastoquinone; PS, photosystem; Qa, first stable photosystem II electron acceptor; TAPS, tris(hydroxymethyl)methylaminopropanesulfonic acid; TPT, triphenyltin chloride; Tricine, *N*-tris(hydroxymethyl)methylglycine.

with the low pH environment, favoring deactivation of the S-states via luminescence-producing back reactions, the result of which is an increase in delayed light emission. We used this technique to probe the equilibration of protons released from the PS I donors, DAD or duroquinone, with the protonatable sites on the OEC. Our data suggest that the catalytic site on the OEC does indeed face the proton-sequestering domains rather than the lumen, and that protons released during PS I-mediated electron transport equilibrate with the OEC via localized pathways which do not include the lumen.

Materials and Methods

Chloroplasts were isolated from market spinach using the method of Ort and Izawa (1973), except the leaf homogenate was pelleted by bringing the centrifuge to 7700 × *g* and then immediately turning it off. The grinding medium contained 300 mM NaCl, 30 mM Na-Tricine at pH 7.8, 3 mM $MgCl_2$, and 0.5 mM Na-EDTA; the wash/resuspension medium contained 200 mM sucrose, 5 mM Na-HEPES at pH 7.5, 2 mM $MgCl_2$, and 0.5 mg BSA/ml. In the experiment of Fig. 4, the "Tricine-soaked" sample was prepared as above, except the wash/resuspension medium was supplemented with 50 mM Na-Tricine at pH 7.5. The chloroplasts were kept on ice in the dark and were used within 3 hr.

All experiments were performed at room temperature. Prompt fluorescence and oxygen evolution were measured as described by Theg *et al.* (1986). Millisecond delayed fluoresence was measured using the rotating-sector phosphoroscope described by Wraight and Crofts (1971). The basic assay medium contained 25 mM Na-Tricine (pH 8.0–8.5) or Na-MES (pH 6.5), 10 mM KCl, 5 mM $MgCl_2$, 0.2 mM DAD, 1 mM Na-ascorbate, 0.1 mM methylviologen, and 20 μg Chl/ml. In two experiments (Figs. 4 and 5), 0.5 mM durohydroquinone replaced the DAD/ascorbate donor system. Further additions are noted in the figure legends.

The general protocol for all delayed fluoresence experiments was as follows: Time (t) = 0; chloroplasts were added to a curvette containing assay medium and then placed in the dark. t = 3 min; two 10 μsec flashes were fired. t = 3 min + 5 s; 5 μM DCMU was added to prevent further S-state turnover. t = 3 min + 15 s; the shutter in front of the actinic light (2.4 mW/cm^2 through a Corning CS4-96 filter) was opened, initiating PS I-dependent proton pumping and forming the luminescence substrate in PS II. This two-flash/DCMU protocol sets the OEC into a state from which the highest proton-dependent delayed fluorescence induction curve can be measured (Bowes and Crofts, 1978).

Except for desaspidin, all chemicals and reagents were obtained from commercial vendors. Desaspidin, which is no longer commercially available, was a kind gift from Dr. G. H. Krause. The more readily available uncoupler that can be removed by BSA addition is CCCP. We avoided this compound, however, because it is an ADRY reagent (Renger, 1972) and is known to inhibit delayed fluorescence emission (Renger *et al.*, 1973; Malkin, 1977).

Results

Characterization of the Delayed Fluorescence Induction

Figure 1 shows representative traces of delayed fluorescence induction curves obtained at pH 8.5 and 6.5. Of several important points to note, the first is that, as shown by Bowes and Crofts (1981), the amplitude of the induction curves is higher at acid than at alkaline pH. For ease of presentation, the alkaline curves in Fig. 1 and all subsequent delayed fluorescence figures have been expanded approximately three times compared to the pH 6.5 curves. Second, the curves are kinetically complex. Earlier work by Crofts and his colleagues (Wraight and Crofts, 1971; Bowes and Crofts, 1978)

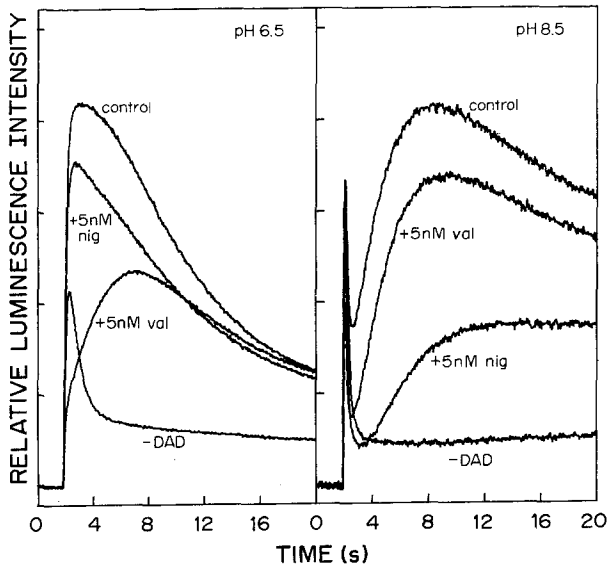

Fig. 1. The effects of nigericin, valinomycin, and DAD on luminescence induction curves at low and high pH. The curves were recorded as described in Materials and Methods. Note that the curves in the right-hand panel were vertically expanded threefold compared with those in the left-hand panel. Nig = nigericin, val = valinomycin.

showed that, following an initial spike at alkaline pH, the rising phases of the delayed fluorescence curves correspond to a shift in the S-state equilibrium toward deactivation in response to a buildup of a protonmotive force (Δp). This point is demonstrated in Fig. 1 by a loss of the rising phase in the absence of DAD. The decay of the curves at longer times is due to the energy-dependent decrease in fluorescence yield (cf. Wraight and Crofts, 1971).

Finally, the respective contribution of the $\Delta\psi$ and ΔpH components of the Δp to the rising phase of the induction curve can be determined by examining the effects of valinomycin and nigericin additions (Bowes and Crofts, 1981). It can be seen that, in the presence of valinomycin and K^+ to inhibit the $\Delta\psi$ component, the ΔpH contributes a slowly rising component that peaks at approximately 8 s. Since we are interested in following the equilibration of protonatable sites on the OEC with protons released during PS I-dependent electron transport, we will concentrate on this slowly rising, ΔpH-dependent component of the induction curves.

Effects of Reversible Uncoupling on the Kinetics of the Delayed Fluorescence Induction Curves

Dilley and Schreiber (1984) showed that the uncouplers, desaspidin and CCCP, could be completely withdrawn from chloroplasts by the addition of BSA. Apparently the BSA binds these compounds and functionally removes them from the solution. Those authors used this technique to remove protons from the membrane-associated domains, and thus recouple the thylakoids for subsequent photophosphorylation measurements.

We wanted to test whether the protonatable sites on the OEC faced the lumen, where the proton concentration in the dark is not affected by uncouplers (Laszlo *et al.*, 1984a), or the domains, from which protons can be removed by uncouplers added in the dark. Thus we monitored the induction of delayed fluorescence before and after exposure of the thylakoids to a reversible uncoupling treatment with desaspidin and BSA. We predicted that there should be no effect of reversible uncoupling on the delayed light induction kinetics if the OEC faced the lumen, i.e., the lumen pH would have been the same in both treatments. On the other hand, if the OEC faced the domains, then the higher initial domain pH caused by proton release with uncoupler addition should delay the rise of the induction curves while the domains are repopulated with protons. Specifically, the induction curves for the "BSA only" and the "BSA, then desaspidin" samples should be identical, but the curve for the "desaspidin, then BSA" sample should be delayed. Furthermore, the domains have been postulated to maintain a pH of approximately 7.0 in the dark for long periods of time regardless of the pH

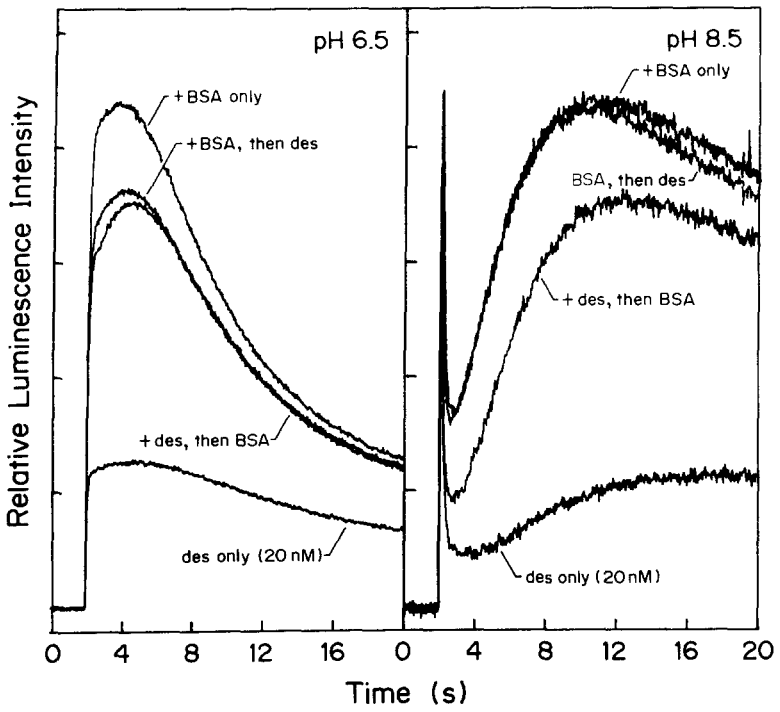

Fig. 2. The effect of reversible uncoupling by 20 nM desaspidin and BSA on the kinetics of the luminescence induction curves at high and low pH. When present, BSA was 1 mg/ml. The protocol described in Materials and Methods was followed, except for the "BSA, then des" and "des, then BSA" samples; the first compound was present at $t = 0$, the second compound was added at $t = 30$ s. Curves in the left- and right-hand panels are plotted on different scales. Des = desaspidin.

at which the chloroplasts are suspended (Theg *et al.*, 1982). Therefore, since added uncouplers will only affect a release of protons from the domains when they are out of equilibrium with those in the external medium, effects of reversible uncoupler treatment on the delayed fluoresence induction kinetics should be apparent only at alkaline pH.

These predictions were tested for two desaspidin concentrations in the experiments shown in Figs. 2 and 3. It can be seen that at alkaline pH, the predictions based on the location of the OEC in the domains were borne out. The "desaspidin, then BSA" delayed light curve obtained from a sample that started with empty domains was delayed compared to the two controls that started with protonated domains. There was very little rise in the "desaspidin only" luminescence curves, again indicating that PS I-dependent proton pumping was responsible for the induction of delayed fluorescence.

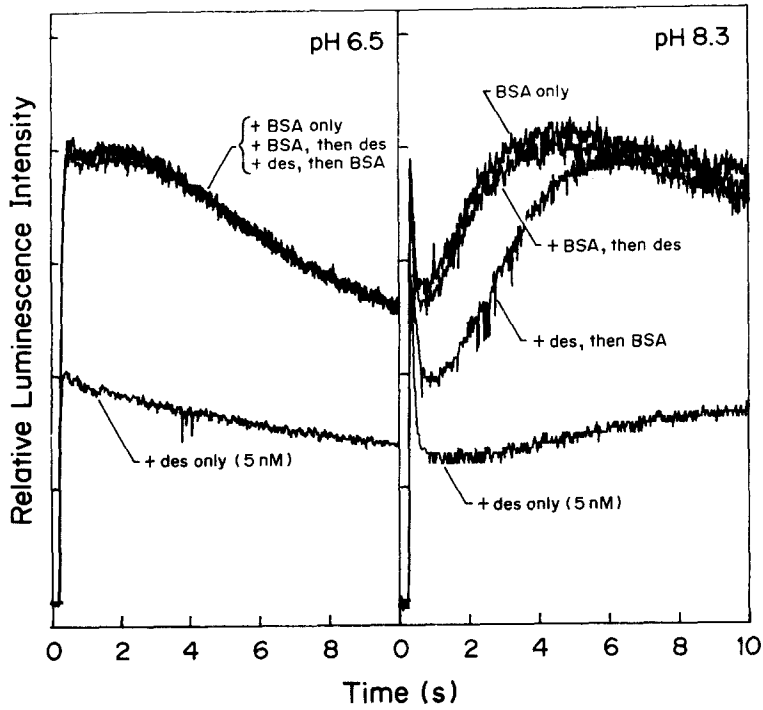

Fig. 3. The effect of reversible uncoupling by 5 nM desaspidin and BSA on the kinetics of the luminescence induction curves at high and low pH. Experimental conditions as in Fig. 2. Des = desaspidin.

At pH 6.5 and in the presence of 20 nM desaspidin, either before or after BSA was added, we found there was some loss of delayed fluorescence intensity (Fig. 2). The reason for this was not clear, but it was obviously not due to reversible uncoupling since the order of addition of desaspidin and BSA was essentially without effect. In a different experiment in which only 5 nM desaspidin was used (Fig. 3), the two control curves were coincident with the "desaspidin, then BSA" curve. From these experiments we conclude that, as predicted if the OEC faces the domains, reversible uncoupling retarded the rise of the delayed light induction curves at alkaline, but not at neutral, pH.

Do Protons Released during PS I-Mediated Electron Transport Travel to the Environment of the OEC through the Bulk Phase in the Lumen?

From experiments reported earlier (Wraight and Crofts, 1971; Bowes and Crofts, 1978) and in this paper, it is clear that the rise in delayed

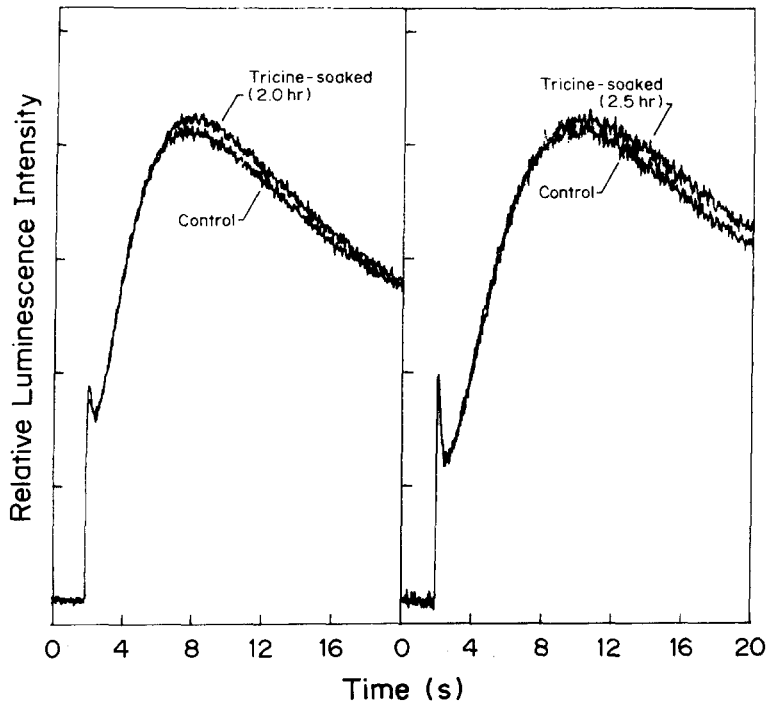

Fig. 4. The effect of increasing the buffering capacity of the thylakoid lumen with Tricine on the kinetics of the luminescence induction curves. "Tricine-soaked" samples were prepared by supplementing the normal chloroplast resuspension medium with 50 mM Na-Tricine (see Materials and Methods). All curves were recorded as in Fig. 1 in the same pH 8.0 assay medium. The electron donors to PS I were DAD/ascorbate in the left-hand panel and DQH_2 in the right-hand panel. Times indicated are those elapsed after Tricine addition.

fluorescence which peaks around 8 s is due to acidification of the environment of the OEC by protons released during PS I-mediated electron transport. We can test whether these protons reach the OEC by traveling through the lumen by looking for a delay in the rise of delayed light caused by increasing the lumen buffering capacity with added buffers. Flores *et al.* (1983) showed that Tricine will equilibrate across the thylakoid membrane with a half-time of approximately 1 hr at 0–4°C. Therefore, we incubated thylakoids in the presence of an additional 50 mM Tricine for 2–2.5 hr and compared their delayed fluoresecence rise kinetics with samples of the same chloroplast preparation stored in normal resuspension medium (5 mM Hepes, Fig. 4). No discernible difference was observed between the curves recorded with control and "Tricine-soaked" samples. By artificially offsetting one of the curves in time compared to the other, we determined that we could have detected a delay as short as 400 ms (not shown). This experiment suggests that the

PS I-derived protons did not pass through the lumen before equilibrating with sites on the OEC.

The two panels in Fig. 4 show delayed fluorescence induction curves obtained with two PS I donor systems, DAD/ascorbate on the left and DQH_2 on the right. The latter should, in principle, be the preferred donor system since it includes electron transport through the PQ pool (Izawa and Pan, 1978). In these experiments, however, we found the luminescence induction curves to be so sensitive to aging of the DQH_2 as to preclude its routine use. Nevertheless, as Fig. 4 illustrates (right-hand panel), we were able to reproduce the general shape of the induction curves using DQH_2 as the PS I donor, and those curves were not retarded by increasing the buffering capacity of the lumen with Tricine.

Effects of CF_0 Inhibitors on Delayed Fluoresence Induction Curves

The experiments of Fig. 4 suggested that the protons released by PS I donors equilibrate with the protonatable sites on the OEC without passing through the lumen. If this is true, then one might postulate that specific pathways exist in the thylakoid membrane over which those protons travel. Such pathways may consist of proton-conducting pores within the membrane (Dilley *et al.*, 1982; Gounaris *et al.*, 1983) or of proton-hopping protein networks (Nagle and Morowitz, 1978). In any case, we postulated that it might be possible to block the diffusion of protons along these putative pathways with compounds that inhibit proton conduction in other, better defined systems.

Proton movement through the CF_0 protein complex of the chloroplast coupling factor is inhibited by DCCD and TPT (McCarty, 1980). Their effects on the induction of delayed fluoresence under our experimental conditions are shown in Fig. 5. It can be seen in the left-hand panel that both inhibitors caused a marked reduction in the rise of the delayed fluorescence intensities at concentrations similar to those required to block CF_0 proton conduction.

Some organometallic compounds of tin and mercury have been shown to uncouple photosynthetic electron transport through a Cl^-/OH^- antiporter mechanism (Whatling-Payne and Selwyn, 1974). The uncoupling activity of these compounds was specific for Cl^- as the exchangable anion (Whatling and Selwyn, 1970; Whatling-Payne and Selwyn, 1974). Although TPT has no known antiporter activity, the possibility remained that its effects on the delayed fluorescence curves was due to a slight uncoupling activity. Therefore, we repeated the last experiment in a medium in which SO_4^{2-} salts replaced the Cl^- salts (right-hand panel). It is clear that TPT still inhibited the rise of the induction curves in the absence of Cl^-, albeit at somewhat higher concentrations.

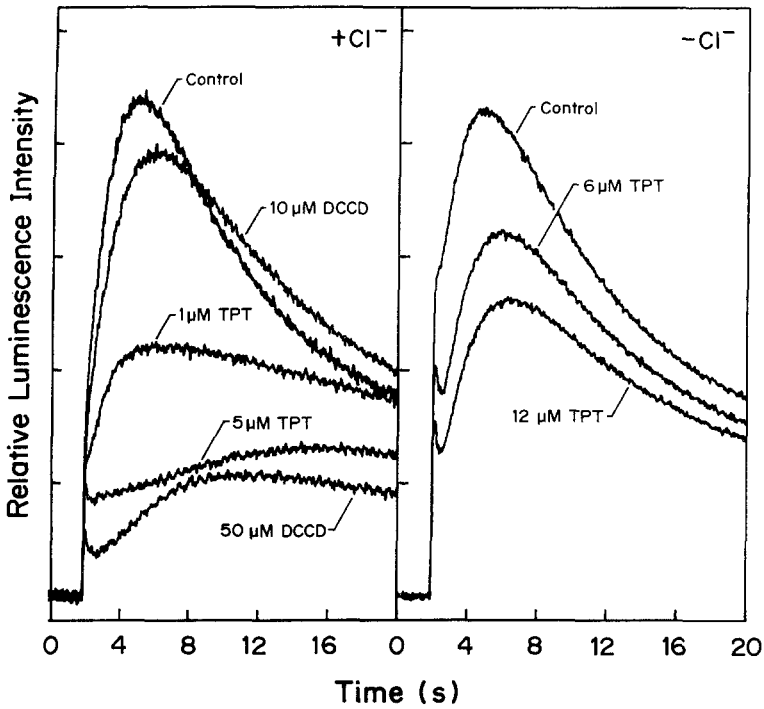

Fig. 5. The effects of DCCD and TPT on the luminescence induction curves. Curves were recorded as in Fig. 1 in pH 8.3 assay media; DQH_2 was the electron donor. In the right-hand panel, 5 mM K_2SO_4 and 5 mM $MgSO_4$ replaced the usual 10 mM KCl and 5 mM $MgCl_2$, respectively. DCCD was added from a methanolic stock prepared that day.

Effects of Desaspidin/BSA, DCCD, and TPT on Electron Transport and the PS II Back Reaction

Delayed fluorescence is, in principle, sensitive to factors affecting the stability of the S-states on the one hand, and the luminescence substrates $P680^+$ and Qa^- on the other. For instance, if the S-state set by the two flash/DCMU protocol deactivated to S_1 more quickly in the presence of, say, DCCD, then subsequent proton pumping by PS I-mediated electron flow would stimulate delayed fluorescence less efficiently because the S_1 to S_2 transition occurs without the release of a proton (cf. Förster and Junge, 1985, and references therein). In the next two experiments described, we tested for such unexpected effects of our different treatments.

If DCCD and TPT inhibited the rise of delayed fluorescence because of heretofore unknown effects on the OEC, we might expect them to inhibit electron transport from this enzyme complex. DCCD has been reported to

Table I. The Effect of DCCD and TPT on Electron Transport[a]

Electron acceptor	Additions	Rate	Percent of control
DMQ/FeCN (+DBMIB)	None	185	100
	50 μM DCCD	159	86
DMQ/FeCN (+DBMIB)	None	184 ± 16	100 ± 9
	100 μM DCCD	156 ± 8	85 ± 5
Methylviologen	None	439 ± 36	100 ± 8
	1 μM TPT	430 ± 37	98 ± 8
	5 μM TPT	406 ± 27	92 ± 6

[a]Chloroplasts (25 μg Chl/ml) were assayed in a pH 8.0 medium containing 100 mM sucrose, 50 mM Na-TAPS, 10 mM KCl, 5 mM $MgCl_2$, 1 μM nigericin, and, when present, 0.5 mM DMQ, 1 μM DBMIB, and 0.5 mM FeCN or 0.1 mM methylviologen. The stock DCCD solution was prepared fresh each day. Rates (± SD) are expressed in μeq/mg chl hr and are an average of two (experiment 1) or three (experiments 2 and 3) determinations.

inhibit electron transport from water to methylviologen, but not from water to the PS II acceptor, DMQ (Sane *et al.*, 1979). TPT, on the other hand, was reported to have little effect on whole-chain electron transport (Gould, 1976). We confirmed these observations under conditions that closely resembled those in our delayed light experiments (Table I). It can be seen that both compounds, when added in the highest concentrations used in Fig. 5, inhibited electron transport out of the OEC by less than 15%. We can conclude, therefore, that these compounds do not block the rise of delayed fluorescence through direct inhibition of the OEC.

By virtue of their ability to donate electrons to the oxidizing side of PS II, ADRY reagents block the back reaction between $P680^+$ and Qa^- and thereby inhibit delayed light emission (Renger *et al.*, 1973; Malkin, 1977). In order to rule out the possibility that this type of effect was responsible for the loss of, or delay in, the rise of the luminescence induction curves, we checked for ADRY-type behavior of DCCD, TPT, and the desaspidin/BSA treatment. With DCMU-poisoned thylakoids, inhibition of the back reaction by ADRY reagents can be readily observed as a deceleration of the return of prompt fluorescence, measured after a light period, to the dark-adapted F_0 level (Cheniae and Martin, 1978; Theg *et al.*, 1986). This is demonstrated for the ADRY compound, CCCP, in Fig. 6 (triangles). Compared to the control (circles), fluorescence remained higher (i.e., $[Qa]_{ox} = (F_{max} - T(t))/(F_{max} - F_0)$ remained lower) during a given dark time interval when CCCP was present. The time courses for the return of the lower fluorescence state in the dark in the presence of DCCD, TPT, or "desaspidin, then BSA" were identical to that of the control (Fig. 6). Thus, none of the treatments that caused profound changes in the rise kinetics of the delayed fluorescence curves could be attributed to an ADRY-type behavior of the elicitors.

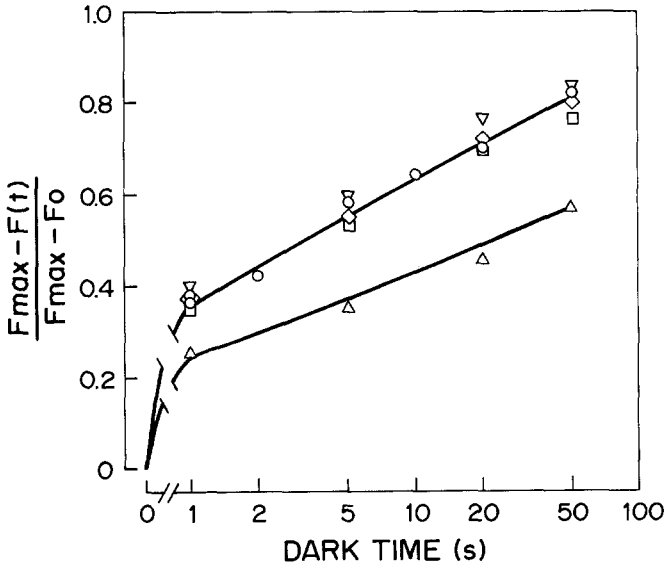

Fig. 6. A test for ADRY-like behavior of DCCD, TPT, and desaspidin/BSA. ○, no additions; △, 0.5 μM CCCP; □, 50 μM DCCD; ▽, 5 μM TPT; ◇, 50 μM desaspidin, followed by 2 mg BSA/ml at t = 15 s. Chloroplasts (10 mg Chl/ml) were added to pH 8.5 assay medium containing 5 μM DCMU at t = 0. t = 1 min; blue actinic light on. t = 1 min, 5 s; actinic light off. t = 1 min, 5 s + dark time; actinic light on again. F_0 and F_{max} are the initial and maximal levels of fluorescence recorded during the first illumination period, respectively; $F(t)$ is the initial level of fluorescence obtained with the second illumination period. F_0 and F_{max} were determined for each point.

Discussion

The experiments in this paper were designed principally to determine whether the catalytic site of the OEC rapidly equilibrates with protons in the thylakoid lumen or in the membrane-associated domains. The delayed light technique we used is particularly suitable for such a study because with DCMU present we can be sure that the protons driving the luminescence increase did not originate from the OEC.

A number of unexpected phenomena were noticed during our investigation which set practical restrictions on the types of experiments we could perform. First, the amplitudes, and to a lesser extent, the shapes, of the delayed fluorescence induction curves were remarkably sensitive to the age of the chloroplast preparation, both decreasing with time. Therefore, all experiments were performed within 3 hr after the leaves were homogenized. Additionally, curves that were to be compared to one another were obtained at as close to the same time as was possible, and no quantitative comparisons

were attempted for curves obtained from different preparations. Second, the induction curves were affected by quite low concentrations of valinomycin and uncouplers (Figs. 2–4). This might have been due, in part, to the low intensity of the blue-green actinic light used, but it is not clear that this accounts for the phenomenon entirely. For instance, a higher concentration of nigericin was required to inhibit steady-state photophosphorylation than to inhibit the rise of delayed fluorescence, even though both were measured under nearly identical conditions (data not shown). Within a single preparation, the luminescence signal became more sensitive toward desaspidin with age.

In spite of the limitations imposed by the phenomena described above, we were able to demonstrate that the delayed light induction curves behaved toward a reversible uncoupler addition as predicted by a model that places the OEC's protonatable groups in equilibrium with protons in the sequestered domains, and not directly exposed to the lumen phase. One possible explanation of these data could be that the desaspidin dissipates a bulk phase to bulk-phase ΔpH imposed by transferring the chloroplasts from the pH 7.5 storage buffer to the pH 8.5 assay medium. Yet the decay of the light-induced proton pump proceeds with a half-time of no more than 10 s at room temperature (cf. Abbott and Dilley, 1983). Therefore, 3 min after the addition of chloroplasts to the assay medium, the time at which the experiments of Figs. 2 and 3 were performed, less than 0.2% of the bulk-phase gradient would remain. On the other hand, protonophoric effects of uncouplers have been observed for as long as 2 hr after transfer of chloroplasts to an alkaline assay medium and kept in darkness (Laszlo *et al.*, 1984a) and have been attributed to their action on a metastable proton pool. Additionally, the effects of desaspidin applied in the dark disappeared at neutral or slightly acid pH, also a behavior observed with all sequestered domain-related phenomena. Where it has been determined, the pK of buffering groups within the domains are between 7.2 and 7.8 (Theg *et al.*, 1982; Theg and Junge, 1983; Laszlo *et al.*, 1984a); the loss of the desaspidin effect at pH 6.5 is compatible with a similar pK controlling proton release in these experiments.

Experiments performed with inside-out thylakoids appear to support the idea that the OEC resides at the membrane/lumen interface. While it is true that this seems to be the location of the three Tris- and salt-releasable polypeptides with molecular weights 32, 23, and 17 kD (Akerlund and Jansson, 1981), it can be argued that these three do not constitute the entire OEC, nor are they completely exposed to the lumen phase. Preparations of thylakoids depleted of all three proteins can still retain the full compliment of the thylakoid Mn (Ono and Inoue, 1983). Even within the set of the three polypeptides there is strong evidence that certain amino groups (Laszlo *et al.*, 1984a) and a binding site for reductants (Ghanotakis *et al.*, 1984a, b) are sterically shielded from the aqueous medium. Furthermore, Bowlby and

Frasch (1986) recently isolated a Mn-containing complex from thylakoids that contains a number of intrinsic proteins in addition to the three extrinsic polypeptides. Finally, it should be stressed that our experiments identify the location of the *catalytic site* of the OEC. It is this site which we suggest is facing the proton-sequestering domains; portions of the complex not involved in the active site could be exposed to the inner lumen.

Our experiments with Tricine, DCCD, and TPT are more difficult to interpret than are the reversible uncoupling experiments. The lack of effect of Tricine on the rise kinetics of the luminescence induction curves suggests that the protons liberated by PS I-mediated electron transport do not pass through the lumen en route to the OEC's environment. This interpretation is based on the assumptions that Tricine reaches the lumen under our conditions, and that an effect of increased buffering capacity of the lumen would be detected in the time frame of our experiments. The first assumption is supported by the work of Flores *et al.* (1983) using chloroplasts isolated according to the same procedure we used here. The second assumption is difficult to assess quantitatively since we have no model with which to simulate the delayed fluorescence rise curves based on internal pH parameters. Nevertheless, a significant delay was observed when extra electron transport was required to overcome a proton deficit resulting from reversible uncoupler addition, which according to earlier estimates was approximately 30 nmol H^+/mg Chl (Baker *et al.*, 1981; Pfister and Homann, 1986). Assuming a thylakoid volume of 10 μl/mg Chl (Flores *et al.*, 1983) and a 2-hr incubation in 50 mM Tricine (Fig. 5), it would take an additional 70 nmol H^+/mg Chl just to reach the pK of the buffer (8.15). Additionally, we used a low actinic light intensity that was able to drive proton accumulation with the water to methylviologen Hill reaction at only 43% of the light-saturated rate (data not shown). Thus it seems reasonable to expect that an easily detectable delay in the luminescence rise curves would have appeared had the Tricine contributed to the buffering capacity of the space in which the PS I-derived protons moved.

The effects of proton-channel inhibitors on the delayed light induction curves also suggest a localized type of proton movement between their sites of release at PS I and rebinding at the OEC. Artifactual effects of DCCD and TPT due to direct inhibition of the OEC, uncoupling, or ADRY effects were ruled out. Furthermore, as far as we know, these two chemically unrelated compounds appear to share only the property of blocking proton conductance through proteinaceous membrane pores. The most straightforward explanation of their effects on our delayed fluorescence signals is that they block conduction pathways used for membrane-associated lateral proton diffusion. Indeed, Sane *et al.* (1979) interpreted their DCCD inhibition of electron transport data as being related to effects on proton conduction pathways.

An effect of DCCD and TPT in our experiments need not mean that CF_0 is involved in determining the shapes of the delayed fluorescence curves. Although these compounds do interact with the CF_0 complex and inhibit proton flux through it, proteins other than the 8-kDa proteolipid have been shown to be labeled by [^{14}C]DCCD (Azzi *et al.*, 1984), some of which are postulated to be cation translocators. Unpublished work in this laboratory has shown that, under somewhat different conditions than used in the present study, DCCD covalently binds to an unidentified 22-kDa thylakoid polypeptide (Millner and Dilley, unpublished results; Atta-Asafo-Adjei and Dilley, unpublished results).

If our interpretations of the experiments with Tricine, DCCD, and TPT are correct, then protons must be confined to specialized pathways that cover relatively large distances. This problem is encountered in all models and descriptions of "localized" ATP synthesis in chloroplasts (Ferguson, 1985; Haraux, 1985; Dilley and Schreiber, 1984; Horner and Moudrianakis, 1983; Hangarter and Ort, 1985), and its solution is difficult to imagine. On the other hand, the data presented here contribute to the growing body of evidence indicating that protons are sequestered in membrane-associated domains in chloroplasts and can move laterally without passing through the lumen. The physical nature of these domains and pathways remains a fascinating problem of chloroplast bioenergetics.

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References

Abbott, M. S., and Dilley, R. A. (1983). *Arch. Biochem. Biophys.* **222**, 95–104.
Akerlund, H.-E., and Jansson, C. (1981). *FEBS Lett.* **124**, 229–232.
Azzi, A., Casey, R. P., and Nalecz, M. J. (1984). *Biochem. Biophys. Acta* **768**, 209–226.
Baker, G. M. (1983). Ph.D. Thesis, Purdue University.
Baker, G. M., Bhatnagar, D., and Dilley, R. A. (1981). *Biochemistry* **20**, 2307–2315.
Barber, J. (1984). *Trends Biochem. Sci.* **9**, 79–80.
Bowes, J. M., and Crofts, A. R. (1978). *Z. Naturforsch.* **33C**, 271–275.
Bowes, J. M., and Crofts, A. R. (1981). *Biochim. Biophys. Acta* **637**, 464–472.
Bowes, J. M., Crofts, A. R., and Itoh, S. (1979). *Biochim. Biophys. Acta* **547**, 336–346.
Bowlby, N. R., and Frasch, W. D. (1986). *Biochemistry* **25**, 1402–1407.
Cheniae, G. M., and Martin, I. F. (1978). *Biochim. Biophys. Acta* **502**, 321–344.
Dilley, R. A. (1986). In *Encyclopedia of Plant Physiology* (Staehelin, L. A., and Arntzen, C. J., eds.), Vol. 19, Springer-Verlag, Berlin, pp. 570–575.

Dilley, R. A., and Schreiber, U. (1984). *J. Bioenerg. Biomembr.* **16**, 173–193.
Dilley, R. A., Baker, G. M., Bhatnagar, D., Millner, P., and Laszlo, J. (1981). In *Energy Coupling in Photosynthesis* (Selman, B., and Selman-Reiner, S., eds.), Elsevier North-Holland, New York, pp. 47–58.
Dilley, R. A., Prochaska, L. J., Baker, G. M., Tandy, N. E., and Millner, P. A. (1982). *Curr. Top. Membr. Transp.* **16**, 345–369.
Diner, B. A., and Joliot, P. (1977). In *Encyclopedia of Plant Physiology* (Trebst, A., and Avron, M., eds.), Vol. 5, Springer-Verlag, Berlin, pp. 187–205.
Ferguson, S. J. (1985). *Biochim. Biophys. Acta* **811**, 47–95.
Flores, S., Graan, T., and Ort, D. R. (1983). *Photobiochem. Photobiophys.* **6**, 293–304.
Förster, V., and Junge, W. (1985). *Photochem. Photobiol.* **41**, 183–190.
Ghanotakis, D. F., Babcock, G. T., and Yocum, C. F. (1984a). *Biochim. Biophys. Acta* **765**, 388–398.
Ghanotakis, D. F., Topper, J. N., and Yocum, C. F. (1984b). *Biochim. Biophys. Acta* **767**, 524–531.
Gould, J. M. (1976). *Eur. J. Biochem.* **62**, 567–575.
Gounaris, K., Brain, A. P. R., Quinn, P. J., and Williams, W. P. (1983). *FEBS Lett.* **153**, 47–52.
Hangarter, R., and Ort, D. R. (1985). *Eur. J. Biochem.* **149**, 503–510.
Haraux, F. (1985). *Physiol. Vég.* **23**, 397–410.
Horner, R. D., and Moudrianakis, E. N. (1983). *J. Biol. Chem.* **258**, 11643–11647.
Izawa, S., and Pan, R. L. (1978). *Biochem. Biophys. Res. Commun.* **83**, 1171–1177.
Laszlo, J. A., Baker, G. M., and Dilley, R. A. (1984a). *J. Bioenerg. Biomembr.* **16**, 37–51.
Laszlo, J. A., Baker, G. M., and Dilley, R. A. (1984b). *Biochim. Biophys. Acta* **764**, 160–169.
Malkin, S. (1977). In *Encyclopedia of Plant Physiology* (Trebst, A., and Avron, M., eds.), Vol. 5, Springer-Verlag, Berlin, pp. 473–491.
McCarty, R. E. (1980). *Methods Enzymol.* **69**, 719–728.
Miller, M., and Cox, R. P. (1983). *FEBS Lett.* **155**, 331–333.
Nagle, J. F., and Morowitz, H. J. (1978). *Proc. Natl. Acad. Sci. USA* **75**, 298–302.
Ono, T.-A., and Inoue, Y. (1983). *FEBS Lett.* **164**, 255–260.
Ort, D. R., and Izawa, S. (1973). *Plant Physiol.* **52**, 595–600.
Pfister, V. R., and Homann, P. H. (1986). *Arch. Biochem. Biophys.* **246**, 525–530.
Polle, A., and Junge, W. (1986). *FEBS Lett.* **198**, 263–267.
Renger, G. (1972). *Biochim. Biophys. Acta* **256**, 428–439.
Renger, G., Bouges-Bocquet, B., nd Delsome, R. (1973). *Biochim. Biophys. Acta* **292**, 796–807.
Sane, P. V., Johanningmeier, U., and Trebst, A. (1979). *FEBS Lett.* **108**, 136–140.
Theg, S. M., and Junge, W. (1983). *Biochim. Biophys. Acta* **723**, 294–307.
Theg, S. M., Johnson, J. D., and Homann, P. H. (1982). *FEBS Lett.* **145**, 25–29.
Theg, S. M., Jursinic, P. A., and Homann, P. H. (1984). *Biochim. Biophys. Acta* **766**, 636–646.
Theg, S. M., Filar, L. J., and Dilley, R. A. (1986). *Biochim. Biophys. Acta* **849**, 104–111.
Whatling, A. S., and Selwyn, M. J. (1970). *FEBS Lett.* **10**, 139–142.
Whatling-Payne, A. S., and Selwyn, M. J. (1974). *Biochem. J.* **142**, 65–74.
Wraight, C. A., and Crofts, A. R. (1971). *Eur. J. Biochem.* **19**, 386–397.