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Site-Specific Interaction of ATPase-Pumped Protons with Photosystem II in Chloroplast Thylakoid Membranes

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Abstract

The chloroplast thylakoid ATPase proton pump-driven H^+ accumulation in the dark was compared to the light-dependent proton pump driven by either photosystem II or I, in regard to the effects of the resultant acidity on chemical modification reactions. The assays used to detect the acidity effects were: (a) the incorporation of [^{3}H]-acetic anhydride into membrane protein $-NH_2$ groups, and (b) the effect of a certain level of that chemical modification on inhibition of photosystem II water oxidation activity. Based on labeling data with [^{3}H]-acetic anhydride, 20–30 nmol · (mg chl)$^{-1}$ of $-NH_3^+$ groups appear to be metastable in the dark in untreated membranes. The term metastable is used because proton leak-inducing treatments in the dark lead to about 20–30 nmol · (mg chl)$^{-1}$ increase in acetic anhydride labeling, probably due to reaction with the $-NH_2$ form of amine groups. Addition of low levels of uncoupler or a brief thermal treatment caused a loss of protons from the membrane equivalent to the increase in acetic anhydride derivatization. The increase in acetic anhydride derivatization caused inhibition of water oxidation activity. Using thermally sensitized membranes, photosystem II but not photosystem I electron transport (each giving a steady-state proton accumulation of about 50 nmol H^+ · (mg chl)$^{-1}$ restored the lower level of acetic anhydride reactivity as in previous results (Baker *et al.*, 1981). In dark-maintained, thermally treated membranes, ATPase activity, i.e., the proton pump associated with it, also restored the lower level of acetic anhydride labeling, and again acetic anhydride no longer inhibited water oxidation. Because photosystem I activity did not elicit this type of response to acetic anhydride, there appears to be a pathway for ATPase pumped protons which allows them to reach a restricted domain, perhaps intramembrane, common with the photosystem II water oxidation mechanism and unavailable to protons pumped by photosystem I. The membrane structure(s) which determines this site specificity is not yet understood.

Key Words: Photosystem II; photosystem II site-specificity; chloroplast membranes; ATPase proton pump; proton processing; intramembrane proton interaction.

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Introduction[2]

Energy transduction in membrane systems such as chloroplasts, mitochondria, and bacteria is believed to involve proton gradients as the intermediary stage in both the ATP synthetase and the ATPase modes (Boyer *et al.*, 1977). The proton gradients may be transmembrane bulk phase to bulk phase (the Mitchell hypothesis) or there may be situations where the protonmotive force may be localized in domains or subsets of the membrane (Williams, 1962). Our recent experiments with chloroplasts suggest some sort of site-specific, localized domains wherein protons released in the photosystem II water oxidation mechanism interact with certain membrane proteins, including a part of the 8-kD CF_0 proton channel protein, in a way that cannot be duplicated by protons derived from PS I-linked redox reactions (Giaquinta *et al.*, 1975; Prochaska and Dilley, 1978*a*, 1978*b*; Baker *et al.*, 1981). Those studies utilized chemical modification reagents as probes of membrane protein responses to either PS I- or PS II-linked proton release reactions.

As background information for this paper, we had shown that dark-adapted chloroplast membranes, suspended in pH 8.6 buffer, were resistant to inhibition of water oxidation by acetic anhydride unless the membranes were made more permeable to proton movement by uncoupler addition or when proton loss was induced by a brief thermal exposure (Baker *et al.*, 1981; Takahama *et al.*, 1977). In the proton-deficient condition, treatment with acetic anhydride inhibited water oxidation activity, with an associated increase in covalent binding of about 25–40 nmol acetyl · (mg chl)$^{-1}$. Direct pH measurement in the dark under similar conditions showed a loss of protons from the membranes of about 30–40 nmol H^+ · (mg chl)$^{-1}$ after addition of low concentrations of gramicidin. Since acetic anhydride reacts only with the unprotonated form of amine groups, the dark-resistant membrane state was suggested to reflect an acidic domain within the membrane, associated with $-NH_3^+$ groups. Uncouplers or brief thermal treatment apparently allow this local acidity to equilibrate with the alkaline external medium. We suggested that this proton "unloading" produced a greater population of unprotonated, acetic anhydride-reactive amine groups, some associated with water oxidation function. Inhibition of water oxidation activity by acetic anhydride correlated with an increased incorporation of acetic anhydride into membrane proteins (Baker *et al.*, 1981). A light-dependent protection against the anhydride

[2]Abbreviations: PS I and PS II, photosystems I and II; Ac_2O, acetic anhydride; NGG, *N*-glycylglycine; DTT, dithiothreitol; MV, methylviologen; HEPES, *N*-2-hydroxyethylpiperazine-N′-2 ethanesulfonic acid; HEPPS, *N*-2-hydroxyethylpiperazine propane sulfonic acid; BSA, bovine serum albumin; DCCD, dicyclohexylcarbodiimide; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DAD, diaminodurene; PYO, pyocyanine; PMS, phenazine methosulfate; DCMU, *N*′-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea.

inhibition was observed in the presence of the low levels of uncoupler that potentiated inhibition in the dark. This protection specifically required the PS II protolytic reaction and was not observed when only PS I H^+ release occurred.

Isolation of membrane proteins showed that the 8-kD CF_0 protein anhydride labeling level (Tandy *et al.*, 1982) was modulated by the same conditions that showed effects of anhydride on water oxidation activity. This site-specific interaction of photosystem II protons with CF_0 via a restricted domain that is not common with the internal aqueous phase, as judged by the nonequivalence of PS I- and PS II-pumped protons, is consistent with Williams' view that the membrane itself may provide a mechanism for energy-coupled proton movement (Williams, 1962, 1978). It is of interest to know if the sequestering of PS II protons provides a unique mechanism for their interaction with the rest of the CF_0 components and with CF_1 complex. If protection against the anhydride inhibition *does* reflect local protonation of amines that are somehow associated with energy transduction, then these protons should not only interact with the CF_0 proton-conducting channel, but should ultimately reach the adenine nucleotide-binding region of CF_1. Carmeli (1970) reported a light- and dithiol-dependent Mg^{2+} ATPase activity in chloroplasts which induced a proton uptake stoichiometry of about 2 H^+/ATP. The question we consider in this paper is whether the ATPase-generated proton pump mimics photosystem II proton release by bestowing protection against the anhydride inhibition, or whether ATPase pumped protons are equivalent to PS I protons by their failure to produce these effects.

Materials and Methods

Chloroplast Preparation

Spinach leaves obtained from a local market or gathered from a controlled-climate facility maintained at 15°C on a 10 hr light–14 hr dark cycle were used to isolate chloroplasts by the method of Ort and Izawa (1973). These chloroplasts were resuspended to give 2 to 3 mg chl/ml in a medium containing 5 mM HEPES–NaOH, pH 7.5, 200 mM sucrose, 2 mM $MgCl_2$, and 0.5 mg defatted BSA/ml. The chlorophyll concentration was determined by the method of Arnon (1949).

Electron and Proton Transport Assays

Actinic light from a 500-W Quartzline projection lamp was heat filtered through a copper sulfate solution before illuminating a water-jacketed cuvette containing chloroplasts. Electron transport was measured with a Clark type

O_2 electrode by following either oxygen uptake (with methylviologen present) or oxygen evolution (with diaminodurene and ferricyanide present). Light-induced pH changes in the external medium were monitored with a semimicro combination Corning pH electrode. Assays were routinely performed at 18–20°C.

ATPase Activation and Assay

Light- and dithiol-dependent Mg^{2+} ATPase activation is described in the treatment regimes below. Activity was measured by following ATPase-induced acidification of the reaction medium using a pH electrode.

Acetic Anhydride Modification

The level of incorporation of [^{3}H]-Ac_2O into membrane proteins was determined by suspending chloroplasts in 10 ml of reaction medium having a composition that is described in Table IA. Acetic anhydride, 7.0 mM, in anhydrous CH_3OH with [^{3}H]-Ac_2O (Amersham-Searle) added to give a specific activity of 5.13×10^3 cpm/nmol was added to chloroplasts in the dark. The anhydride treatment regime was as described in Table IA. After the samples were quenched with NGG, they were placed on ice and then centrifuged at 20,000 × *g* for 5 min. Each pellet resulting from a 10-ml suspension was resuspended in 10 ml of 50 mM NGG and centrifuged again at 20,000 × *g* for 5 min. The pellets were then extracted twice with 90% acetone and finally centrifuged at 12,500 × *g* for 5 min, giving a protein sediment that was resuspended in 0.5 ml of 5% sodium dodecyl sulfate ($NaDodSO_4$). Following a 2-hr incubation at 50°C, 0.15 ml of the $NaDodSO_4$ solution was transferred to 10 ml of Tritosol liquid scintillator (Fricke, 1975). The remainder of the solution was assayed for protein by a modified procedure of Lowry *et al.* (1951).

Table IA. Preillumination of Thermally Treated Chloroplasts Protects PS II against Ac_2O Inhibition

Preillumination time with MV present	Hill reaction activity after Ac_2O, μeq · (hr · mg chl)$^{-1}$
0 sec	274
5 sec	462
15 sec	462
60 sec	462

Table IB. ATPase Protects PS II against Acetic Anhydride Inhibition

Additions	Hill reaction activity after Ac_2O, μeq · (hr · mg chl)$^{-1}$
2.0 mM ADP	254
2.0 mM ATP	624
50 μM DCCD, then 2.0 mM ATP	140 (220)[a]

[a]This represents the estimated rate had DCCD not inhibited basal electron transport.

Treatment Regimes Used in Tables IA–III

Table IA. Chloroplasts equivalent to 200 μg of chlorophyll were suspended in 2 ml of a reaction mixture containing 50 mM HEPPS–NaOH (pH 8.6), 50 mM KCl, 2 mM $MgCl_2$, 100 mM sucrose, 0.5 mM MV, 3 mM DTT, and 6 mM NaH_2PO_4. The suspension was illuminated at 20°C with saturating white light for 3 min and then transferred, in the dark, to 8 ml of the same reaction mixture (but without MV, DTT, and P_i), at 30.5°C, and incubated at this temperature for 20 sec. The suspension was then cooled to 20°C within 30 sec and a 2 ml aliquot was transferred to an oxygen-electrode cuvette, and 0.5 mM MV was added. The suspension was then either maintained in the dark or illuminated for 5, 15, or 60 sec. One minute after the illumination or after a comparable time in the dark, the samples were treated with 7.0 mM Ac_2O for 30 sec, at which time 50 mM NGG was added to quench the unreacted Ac_2O. After quenching for 45 sec, water $\rightarrow$ MV electron transfer activity was measured, with 5 μM gramicidin added to ensure that all rates were maximally uncoupled.

Table IB. Treatment regime was identical to that described in Table IA, except that 2 mM ATP or ADP was added to the thermally treated suspension, and the adenine nucleotide was present for 1 min under dark conditions before the addition of Ac_2O. The uncoupled ATPase activity following ATP addition was 82 μmol $H^+ \cdot (\text{hr} \cdot \text{mg chl})^{-1}$ at pH 8.6. No activity was detected when ADP was added. As a control, 50 μM DCCD was added to a thermally treated suspension and allowed to incubate for 1 min before the addition of 2 mM ATP. No ATPase activity was found in this sample.

Table II. The reaction mixture used for the thermal treatments in this experiment consisted of 50 mM HEPPS–NaOH, pH 8.6, 0.1 M sucrose, 50 mM KCl, 2 mM $MgCl_2$, and 1.2 mM NaH_2PO_4. Chloroplasts equivalent to 200 μg chlorophyll were added to this reaction mixture at 30°C, maintained at this temperature for 15 sec, and then cooled in an ice water bath to 19°C within 30 sec. Treatments of the resulting 10-ml suspensions were as follows: (1) Photosystem II + I light conditions: to the suspension were added 30 μM PYO and 0.6 mM DTT followed by subsaturating white light that gave a net proton accumulation of 52 nmol $H^+ \cdot (\text{mg chl})^{-1}$ at pH 8.6. All proton uptake assays given in this table were measured separately at pH 8.6 using a 0.5 mM buffer system. Fifteen seconds after illumination began, the suspension was treated with 3.5 mM Ac_2O for 30 sec and the unreacted anhydride was then quenched with the addition of 50 mM NGG. Thirty seconds after this addition, the light was turned off and the sample was placed on ice. (2) Photosystem I light conditions: to a suspension prepared as in (1), 2 μM DCMU was added followed by saturating white light that gave a steady-state proton accumulation of 60 nmol $H^+ \cdot (\text{mg chl})^{-1}$. Ac_2O treatment was

Table II. Photosystem II but not Photosystem I Electron Transport Protects Water Oxidation against Acetic Anhydride Inhibition in Thermally Treated Chloroplasts

Conditions present during Ac_2O treatment of thermally treated membranes	Steady-state proton accumulation nmol H^+ · (mg chl)$^{-1}$, or photophosphorylation rate, μmol H^+ · (hr · mg chl)$^{-1}$, before Ac_2O addition	Water → diaminodurene or water → methyl viologen activity remaining after Ac_2O treatment, μeq · (hr · mg chl)$^{-1}$
1. White light, PS II + I + PYO + DTT	52	760[c]
2. White light, PSI only + PYO + DTT + DCMU	60	300[c] (508)[b]
3. White light, PS II + I + PMS	35 (69)[a]	902[d]
4. White Light, PS I only + PMS + DBMIB	40 (82)[a]	544[d]

[a] Photophosphorylation rates measured with a pH electrode at pH 8.5 in thermally treated membranes at 10^5 erg · cm^{-2} · sec^{-1} for the PS II + I case (PMS only) and at 10^6 erg · cm^{-2} · sec^{-1} for the PS I only case (PMS + DBMIB).

[b] This represents the estimated rate if all of the DCMU had partitioned out during the washing and resuspension procedure.

[c] Water → methyl viologen assay.

[d] Water → diaminodurene assay.

as in (1). Suspensions from (1) and (2) were centrifuged at 37,000 × *g* for 1 min and resuspended in 10 ml of a medium containing 5 mM HEPES–NaOH, pH 7.5, 0.2 M sucrose, 2 mM $MgCl_2$, and 0.5 mg defatted BSA/ml. The centrifugation step was repeated and each pellet resuspended in 0.3 ml of the same medium. Water → MV (0.5 mM) electron transport was then measured as O_2 uptake in the same medium used for the thermal treatments. Using this centrifugation and resuspension procedure, the extent of recovery of H_2O → MV electron transport was determined to be 59% by comparing the activity of a nonthermally treated, nonanhydride-treated suspension that had 2 μM DCMU present [600 μeq · (hr · mg chl)$^{-1}$] with a control that had no DCMU [1020 μeq · (hr · mg chl)$^{-1}$]. The parenthetical rate given in (2) reflects this correction. All rates were maximally uncoupled with the addition of 1.0 μM gramicidin. (3) Photosystem II + I light conditions. A suspension to which was added 30 μM PMS was illuminated with subsaturating white light and treated with Ac_2O as in (1). Following the illumination period, 2 μM DBMIB and then 0.6 mM DTT were added to the suspension before placing on ice. (4) Photosystem I light conditions: 2 μM DBMIB was added to this suspension in addition to 30 μM PMS. The membranes were illuminated with saturating white light and treated with Ac_2O as in (1). Following the illumination period, 0.6 mM DTT was added to the suspension before placing on ice. Both suspensions were centrifuged at 20,000 × *g* for 5 min and each pellet resuspended in 0.2 ml of the same medium used to resuspend samples

(1) and (2). Water → DAD_{ox} (0.5 mM DAD + 1.5 mM ferricyanide) electron transport was then measured as O_2 evolution in the same medium used for the thermal treatments, except at pH 8.0. All rates showed no uncoupler sensitivity and were completely inhibited by 5 μM DCMU. DTT was added at the end of the light treatments in (3) and (4) in order to reverse a severe inhibition of water → DAD_{ox} electron transport by DBMIB. In membranes that were neither thermally treated nor anhydride treated, 2 μM DBMIB inhibited H_2O → DAD_{ox} activity by 70% at pH 8.0. Addition of 0.6 mM DTT completely reversed this inhibition, and the rate obtained was entirely sensitive to 5 μM DCMU.

Table III. Chloroplasts were ATPase-activated and thermally treated as described in Table IA. Experiment 1 shows that light-induced electron transport lasting 5 sec restores a lower level of [^{3}H]-Ac_2O reactivity concomitant with bestowing protection against water-oxidation inhibition. In experiment 2, the chloroplasts were kept in the dark and given either 2 mM AMP (no ATPase activity) or 2 mM ATP to elicit the ATPase H^+ pump prior to giving 7 mM Ac_2O treatment. The protocol follows that given in Table IB. The labeling procedure with [^{3}H]-Ac_2O is described in Materials and Methods.

Results

Electron Transport Compared to ATPase Proton Pump

To ask if the proton pump linked to ATPase activity can protect against the anhydride inhibition of water oxidation activity would seem to involve simply incubating uncoupler-sensitized thylakoid membranes with Ac_2O during ATPase-induced proton accumulation, analogous to the way we conduct light-protection experiments, and then finally assaying for the effect of this treatment regime on water to methylviologen electron transport. However, this approach is prevented by the observation that Ac_2O causes a rapid decay of the ATPase activity, as indicated by the time course of proton-gradient collapse, assayed by 9-aminoacridine fluorescence increase

Table III. ATPase Activity Restores the Lower Level of Ac_2O Binding in Thermally Treated Chloroplasts

Treatment	Hill reaction activity remaining after Ac_2O treatment, μeq · (hr · mg chl)$^{-1}$	Nanomoles acetyl bound per milligram protein
1. Dark	271	98 ± 4
Light	400	85 ± 4
2. AMP	264	106 ± 9
ATP	443	84 ± 9

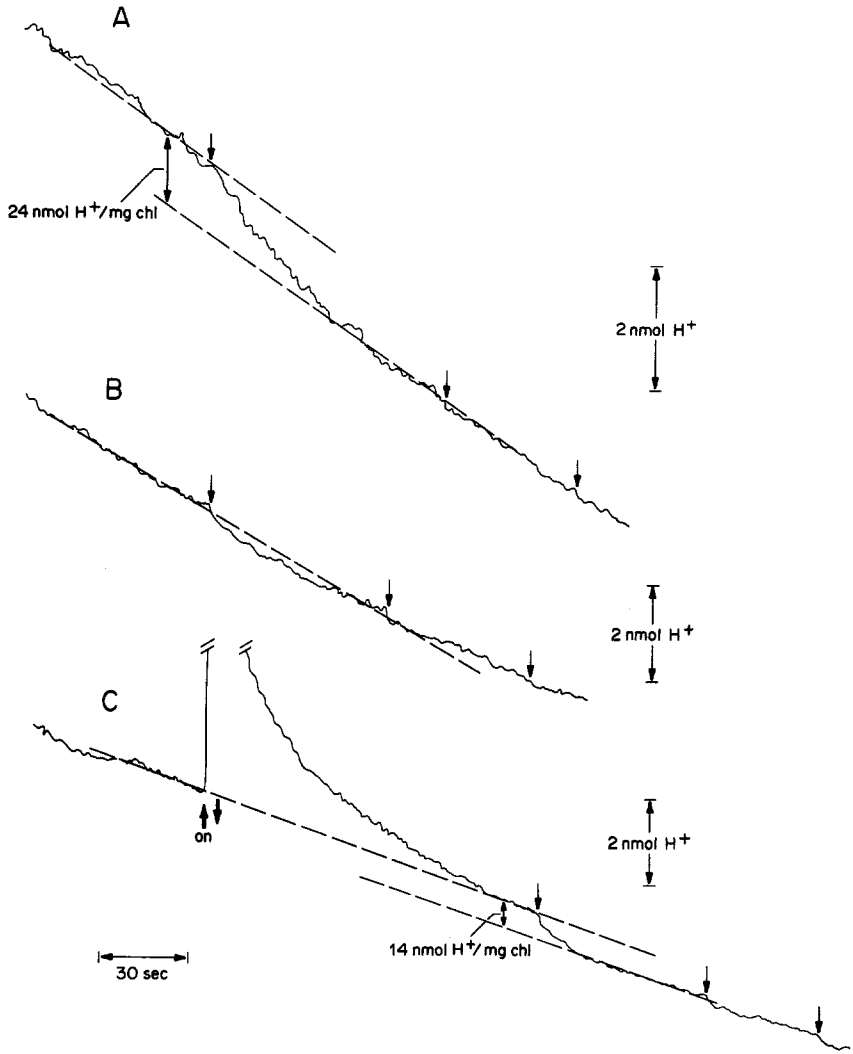

Fig. 1. Chloroplasts were resuspended in a medium containing 5 mM HEPES–NaOH, pH 7.5, 0.2 M sucrose, 2 mM $MgCl_2$, and 0.5 mg BSA/ml. The reaction medium for all pH assays consisted of 0.3 mM HEPPS, titrated to the appropriate pH, as indicated below, 0.1 M sucrose, 50 mM KCl, 2 mM $MgCl_2$, and 0.5 mM MV. Nitrogen gas was circulated above the reaction chamber in order to minimize acid drift due to bicarbonate ion formation. The downward arrows indicate the additions of 1.0 μM gramicidin (1 μl of a 2 mM stock in 2.5 ml reaction medium). (A) This shows uncoupler-induced H^+ efflux from dark-maintained chloroplast membranes that were resistant to water-oxidation inhibition by Ac_2O. To assay for anhydride resistance, chloroplasts were suspended in a medium identical to that used for the pH assays, except that 50 mM HEPPS–NaOH, pH 8.6, replaced the 0.3 mM buffer. Final chlorophyll concentration was 20 μg/ml. Treatment of these membranes with 7.0 mM Ac_2O occurred in the dark for 30 sec, either in the presence or absence of 1.0 μM gramicidin. At the end of this time, the unreacted

following the addition of Ac_2O to an illuminated chloroplast suspension (data not shown). We therefore required an alternative protocol to test for any ATPase-induced protection.

In our previous work (Baker *et al.*, 1981), we showed that exposure of dark-adapted membranes to low concentrations of uncouplers or to a brief thermal transition (from 20 to 30°C for 15–20 sec and back to 20°C), and then treating those membranes with Ac_2O, induced an increased level of [^{3}H]-acetyl incorporation with a concomitant inhibition of water oxidation activity. Membranes that were not thermally treated but incubated with Ac_2O were resistant to this inhibition and showed less covalent derivatization of membrane amine groups. Our working hypothesis proposes an array of buried $-NH_2$ groups (behind the permeability barrier) associated with membrane proteins, in equilibrium with free protons; i.e., $-NH_2 + H^+ \rightleftharpoons NH_3^+$, such that either uncouplers or the thermal treatment, under our conditions, dissipate the H^+ gradient and lead to the formation of more of the $-NH_2$ form. Additional, more direct, evidence that the thermal treatment we use causes a dissipation of an otherwise metastable proton pool is obtained from pH measurements (in the dark) following addition of the uncoupler gramicidin. Figure 1A shows the uncoupler-induced H^+ efflux of about 24 nmol · (mg chl)$^{-1}$ from control membranes kept in darkness. Thermal treatment of another

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anhydride was quenched by adding 50 mM NGG. We waited 45 sec and then illuminated the suspension to measure $H_2O \rightarrow$ MV electron transport activity. A rate of 133 μeq e^- · (hr · mg chl)$^{-1}$ was observed when gramicidin was present during the Ac_2O treatment, compared with 250 μeq e^- · (hr · mg chl)$^{-1}$ obtained when gramicidin was absent. Thermally treated membranes were used in cases (B) and (C) and were prepared as follows. The 0.3 mM buffer medium described above was titrated to pH 8.80–8.85 and then brought to 30°C. Chloroplasts equivalent to 200 μg chlorophyll were then added rapidly to this medium (which decreased the pH to about 8.5), maintained at 30°C for 15 sec, and then cooled to 18°C within 20–30 sec using an ice-water bath. A 2.5-ml aliquot was then transferred to a pH cuvette and 0.5 mM MV was added. Acetic anhydride treatments were performed on similarly thermally treated suspensions in which the 50 mM buffered medium described above was used. After the thermal treatment was complete, a 2-ml aliquot was transferred to an O_2 cuvette, 0.5 mM MV was added, and the suspension was either maintained in the dark or illuminated for 2 sec. After 2 min from the time the light went on, or after a comparable length of time in the dark-maintained case, the membranes were treated with Ac_2O and then quenched according to the procedure in (A). The $H_2O \rightarrow$ MV electron transport activity was then assayed as O_2 uptake, with the observed rates reported below. All rates were maximally uncoupled by further additions of gramicidin where necessary. (B) This represents the effect of uncoupler on thermally treated membranes that were maintained in the dark. Acetic anhydride treatment of those membranes gave a $H_2O \rightarrow$ MV electron transport activity of 117 μeq e^- · (hr · mg chl)$^{-1}$. (C) This trace shows the effect of a 2-sec illumination given to thermally treated membranes on the restoration of uncoupler-induced H^+ efflux measured 117 sec after the light was turned off. The 2-sec illumination accumulated 163 nmol H^+ · (mg chl)$^{-1}$ and decayed with a $t_{1/2}$ of 15 sec. The first addition of uncoupler did not occur until after 8 half-times, when the decay is predicted to be 99.6% complete (<1 nmol H^+/ml chl expected). The $H_2O \rightarrow$ MV electron transport rate observed after Ac_2O treatment in these illuminated chloroplasts was 200 μeq e^- · (hr · mg chl)$^{-1}$ compared with 117 μeq e^- · (hr · mg chl)$^{-1}$ obtained with dark-maintained, thermally treated membranes [case (B)].

sample prior to gramicidin addition resulted in no H^+ efflux (Fig. 1B). A third sample, also thermally treated, when given a brief illumination, once again showed an uncoupler-induced H^+ efflux of 14 nmol · $(mg\ chl)^{-1}$ (Fig. 1C). As indicated in the legend to Fig. 1, sufficient time elapsed after the light was turned off and before addition of the gramicidin to permit >99% loss of the transmembrane proton gradient. Less than 1 nmol H^+ · $(mg\ chl)^{-1}$ would be expected in the transmembrane gradient.

These results are consistent with the Ac_2O effects on water oxidation shown in Table IA, and are in accord with the role of $-NH_2$ groups both in acting as buffering groups for the "sequestered proton pool" (cf. Baker *et al.*, 1981) and for some of those $-NH_2$ to be closely associated with the water oxidation function. Table IA shows that the thermal treatment renders the membranes sensitive to Ac_2O inhibition of the $H_2O \rightarrow MV$ Hill reaction. When the thermally treated membranes were illuminated in the presence of methylviologen *prior* to the addition of Ac_2O, restoration of the protected state of water oxidation activity occurred. If membranes were thermally treated but not anhydride treated, a water $\rightarrow$ MV activity of 490 μeq · $(hr \cdot mg\ chl)^{-1}$ was observed, indicating a complete restoration of the protected state of the water oxidation mechanism by a 5-sec or longer illumination. Membranes that were not thermally treated or anhydride treated generated 702 μeq · $(hr \cdot mg\ chl)^{-1}$. Steady-state electron transport typically showed about 30% inhibition after thermal treating.

These results form the basis of the experimental protocol that will allow us to determine if the ATPase proton pump can mimic the PS II effect of restoring the protected state, i.e., restoring a localized, PS II-specific set of amine groups to the protonated state, unreactive with acetic anhydride. Thylakoids were activated for ATPase function and thermally treated as in Table IA; ATPase activity was initiated by adding ATP, and the membranes were then exposed to Ac_2O. The ATPase activation was carried out by illuminating a suspension of chloroplasts (200 μg chl in 2 ml) with saturating white light for 3 min in the presence of MV and the reductant, dithiothreitol. In order to preserve ATPase activity during subsequent handling, 6 mM orthophosphate was present during the activation and subsequent steps. The suspension was transferred in the dark to 8 ml of reaction medium at 30.5°C to give a final chlorophyll concentration of 20 $\mu g \cdot ml^{-1}$. The use of somewhat higher temperatures, or much longer times than 20 sec at 30.5°C, prevented the restoration of the protected state when those thermally treated membranes were illuminated with MV present. Phosphorylation ($H_2O \rightarrow MV$) was also greatly reduced when the higher temperatures were used, even though high rates of electron transfer activity were still observed (data not shown) in nonanhydride-treated controls. Dilution of the chloroplasts into 8 ml of reaction medium gave an optimum chlorophyll concentration for subsequent

treatments with Ac_2O and a 5-fold dilution of the 3 mM DTT that was present for light-activating the ATPase. High concentrations of DTT will quench Ac_2O, and the dilution to 0.6 mM reduces this effect.

Addition of 2 mM ATP to an ATPase-activated, thermally treated chloroplast suspension produced a gramicidin-uncoupled ATPase activity of 70–100 μmol $H^+ \cdot (hr \cdot mg\ chl)^{-1}$, which was linear for the one minute that was routinely provided before adding Ac_2O. Addition of 2 mM ADP or AMP produced no detectable activity. It should be noted that uncoupler was never used to stimulate ATPase activity during the period prior to anhydride addition. Uncoupled ATPase activities were always assayed in separate experiments.

If the ATPase proton pumping activity can restore local acidity in thermally treated membranes, then subsequent use of Ac_2O, as an assay for this acidity, should reveal protection against inhibition of water oxidation function when compared to an ADP (in place of ATP) control treatment. Analogous to the effect of preillumination, the data in Table IB show that ATPase activity *did* restore the protected state in membranes made sensitive to Ac_2O by prior thermal treatment. An electron transfer rate of 254 μeq $\cdot$ $(hr \cdot mg\ chl)^{-1}$ in the ADP control case reflects the inhibitory effect of Ac_2O on water oxidation activity, and should be compared with the rate of 624 μeq $\cdot$ $(hr \cdot mg\ chl)^{-1}$ obtained when ATP, instead of ADP, was added prior to Ac_2O treatment. Treatment of thermally sensitized membranes with DCCD fully inhibited ATPase activity, and subsequent treatment of these membranes with Ac_2O demonstrated no protection when finally assayed for water oxidation activity, even after correcting the observed rate for the inhibitory effect of DCCD on basal electron transfer.

The effect of the thermal treatment on thylakoid membranes must be further assessed before interpreting these results. Our previous interpretation was that the failure of PS I proton accumulation to fully protect the water oxidation system against anhydride inhibition in uncoupler-sensitized, *non*-thermally treated membranes reflected a permeability barrier to proton access to the PS II centers. Thermal treatment may lower this barrier in some way and allow protons that are accumulated internally to freely reach what would otherwise be a sequestered PS II domain. In view of the ATPase-mediated protection of H_2O oxidation against Ac_2O inhibition, this becomes an important point to test.

The approach for this experiment was similar to that reported earlier (Baker *et al.*, 1981), except there, we used uncoupler to sensitize the membranes to anhydride inhibition. That protocol was to add the anhydride during PS I energization (using DCMU as the inhibitor of PS II function), partition out the DCMU using centrifugation and resuspension steps, and then measure water $\rightarrow$ MV electron transport. The same procedure was

performed on thermally treated membranes, and the results are presented in Table II (compare treatments 1 and 2). Treatment 2 shows that when PS I alone gave a proton accumulation of 60 nmol H^+ · (mg chl)$^{-1}$ in thermally sensitized membranes, there was, as before (Baker *et al.*, 1981), no protection of the water oxidation mechanism against anhydride inhibition. Protection required PS II activity [compare 760 μeq · (hr · mg chl)$^{-1}$ with 508]. To ensure that the lack of PS I-mediated protection that we observed in this experiment was not somehow specific to the use of DCMU as an inhibitor, we also used DBMIB with PMS as a PS I only partial reaction. Jagendorf and Margulies (1960) showed that white light acts as a nonenzymatic reductant of PMS, thus overcoming the need to use a dithiol reagent, as with the pyocyanine cofactor. This becomes an important point in view of the well-established reversal of DBMIB inhibition by dithiols (Reimer and Trebst, 1976; Guikema and Yocum, 1978). For the PS II effect, DBMIB was omitted, allowing PS II activity to occur in addition to the PMS-catalyzed PS I reaction. In both cases, the final assay for PS II water oxidation activity was a Class III reaction, water → diaminodurene with excess ferricyanide present. As shown in Table II, protection against the anhydride inhibition again required PS II activity [compare 902 μeq · (hr · mg chl)$^{-1}$, treatment 3, with 544, treatment 4), even though the proton accumulation occurring in the PS II plus PS I case was comparable to that generated by PS I alone.[3]

The failure of PS I activity to bestow protection suggests that, under our conditions, the presence of 2 μM DBMIB had little capacity to act as a Class III electron acceptor. In a separate, control experiment, we observed less than 30 μeq · (hr · mg chl)$^{-1}$ of water → ferricyanide electron transport with 2 μM DBMIB present.

We have indicated above that PS I activity afforded no protection, rather than stating that some protection was conferred. This is indicated by comparing the inhibition observed under PS I only treatment conditions (comparing treatment 4 with 3 in Table II shows 40% inhibition) with the pattern obtained in the following control experiments. When the same thermally treated membranes were given a 5-sec illumination (with MV present) prior to anhydride treatment, a measure of H_2O → MV electron transport showed 500 μeq · (hr · mg chl)$^{-1}$. The protocol followed that given

[3]In the experiments using PMS ± DBMIB, we noted that if 0.6 mM DTT was present during the acetic anhydride treatment, both cases (plus or minus DBMIB) showed protection against the anhydride inhibition of water oxidation. When the PMS plus DBMIB case was assayed finally for H_2O → DAD_{ox} electron transport, an activity of 740 μeq · (hr · mg chl)$^{-1}$ was observed compared to 702 μeq · (hr · mg chl)$^{-1}$ for the PMS minus DBMIB case. As observed, no difference in the rates is expected if DTT were allowing PS II recovery with DBMIB present. In this connection, separate experiments demonstrated the recovery of a DCMU-sensitive component of PMS-mediated photophosphorylation when 0.6 mM DTT was present (data not shown).

in Table IA. Dark-maintained membranes generated only 300 μeq · (hr · mg chl)$^{-1}$ after anhydride treatment. The magnitude of this inhibition was 40%, identical to the extent of inhibition observed when only PMS-mediated PS I was active. Thermally treated but nonanhydride treated membranes showed an activity of 500 μeq · (hr · mg chl)$^{-1}$, indicating complete protection against anhydride inhibition by the 5-sec illumination. If membranes were neither thermally treated nor anhydride treated, a rate of 725 μeq · (hr · mg chl)$^{-1}$ was observed, demonstrating that the thermal treatment alone inhibited steady-state electron transport ($H_2O \rightarrow MV$) by about 30%.

The results in Table II lend added significance to the ATPase-mediated protection and suggest that protons released by water oxidation and protons released by the ATPase proton pump can reach a common "domain" that cannot be protonated by PS I-linked protons. Ultimately, protons from all three pump sources reach the inner aqueous space, but there seems to be additional, restricted domains for proton interactions with the membrane.

Effects of Labeling of Membrane Proteins

If the sensitivity of thermally treated membranes to Ac_2O reflects the availability of the uncharged, anhydride-reactive form of a critical group of amines, then the effect of preillumination ($H_2O \rightarrow MV$), or ATPase proton pumping, should not only be to protect the water oxidation mechanism by restoring the protonated state of these amines, but also to reduce the level of anhydride incorporation, a diagnostic assay for the unprotonated form of amines. Table III shows that either preillumination or ATPase activity restored the lower level of anhydride binding concurrent with the restoration of the protected state of water oxidation. The notion of the protonation state of the amine serving to modulate the anhydride effect on thylakoid membranes remains consistent.

Discussion

As already indicated, Baker *et al.* (1981) provided evidence suggesting that the level of covalent anhydride binding is modulated by local acidification of a critical group of amines that interact specifically with the protons released by the water oxidation mechanism. When uncoupler treatment sensitized dark-adapted membranes to Ac_2O inhibition, restoration of the protected state required a continuous, PS II-dependent proton flux. A brief thermal exposure mimics uncoupler treatment, but an important difference is that a brief illumination (with MV present) is sufficient to restore the Ac_2O-*insensitive* state that existed in membranes prior to thermal treatment (Tables

IA and III). Resistance to anhydride inhibition persisted during subsequent dark periods, consistent with the presence of a metastable "pool" of protons in equilibrium with an array of $-NH_2$ groups within a restricted domain.

Measurement of H^+ efflux with a pH meter in response to addition of uncoupler in the control (not thermally treated) chloroplasts, but no such H^+ efflux from thermally treated membranes, showed directly (Fig. 1) the presence and absence, respectively, of the metastable proton pool behind the membrane permeability barrier. Illumination of thermally treated membranes partially restored the dark, uncoupler-induced H^+ efflux. We interpret these data as due to the PS II activity restoring the protonated state of certain amine groups of intrinsic membrane proteins, some associated with water oxidation, as discussed by Baker *et al.* (1981). As observed in the earlier work, this protection required PS II activity since, as seen in Table II, thermally treated membranes do not experience a restoration of the protected state when only PS I electron and proton transport occurred.

Although the PS I proton pump activity failed to protect the water oxidation mechanism against Ac_2O, the ATPase-dependent proton pump, functioning in the dark, did restore the protected state (Tables IB and III). Moreover, the ATPase proton pump conditions led to the lower level of acetic anhydride labeling (Table III), similar to the PS II activity effect. These results suggest that ATPase activity pumps protons into a PS II-specific domain that is not accessible to PS I protons. Any reverse electron transport that is associated with ATPase activity (Rienits *et al.*, 1973) is not likely to account for the protection since the Ac_2O effect is not dependent primarily on PS II redox activity (see Table II in Baker *et al.*, 1981) but, rather, on the acidity present behind a postulated permeability barrier. The same argument would also mean that purely electronic effects cannot explain the failure of PS I protons to protect the water oxidation mechanism from anhydride inhibition in thermally treated membranes (Table II).

The protection of PS II activity by the ATPase proton pump under conditions where PS I proton accumulation does not protect suggests a close functional and structural proximity between the energy-coupling complex and the PS II water-oxidizing proteins. This association may reflect random, but close, interactions of coupling factors with PS II units, or PS I units, such that when a given coupling factor is closely associated with a PS II unit it responds to PS II proton release in a site-specific manner. Conversely, when such a PS II–CF_0–CF_1 interaction state experiences ATPase proton pumping, those protons may initially be released into a PS II-specific domain. This would require that those CF_0–CF_1 complexes associated more closely with PS I (or not associated with either photosystem) would pump protons either directly into a PS I-specific domain, or into the inner aqueous space. Evidence from

structural analysis, primarily by freeze-fracture techniques, indicates that the CF_0–CF_1 complexes, and the large freeze-fracture-revealed particle believed to be associated with the PS II reaction center (Arntzen *et al.*, 1969), are laterally mobile (Staehelin, 1976). Lateral mobility could lead to transient, close association of a given CF_0–CF_1 complex with a PS II unit, or a PS I unit.

An alternative explanation of the data is that there may be regions of the membrane that contain predominantly PS II units, having a set of photosystem II-associated CF_0–CF_1 complexes. It has been suggested that the appressed regions of grana thylakoids contain mostly PS II units, with PS I units enriched at the edges of the grana stacks and in the stroma thylakoids (Andersson and Anderson, 1980). The evidence of Staehelin (1976) would thus confine the CF_1 to those thylakoid regions which could be far from some of the PS II units. If PS II units and coupling factor complexes are spatially separate, then a problem faced by our model is that local acidity due to sequestered PS II proton release may need to extend over rather large distances to include the water-oxidizing proteins as well as the energy-coupling complex. However, specialized H^+ conducting structures cannot be ruled out and the views of Nagle and Morowitz (1978) offer a possible mechanism for such specific proton-conducting devices along proteins.

Both of the above suggestions are speculative at present, but they are possible directions for future work. Nor is it at all clear whether the postulated restricted domain is a "buried" structure, within the membrane, perhaps provided by integral membrane proteins, or an interfacial phenomenon involving proteins at the surface of the membrane (Kell, 1979). What is evident is that the PS II proton release event shares a common functional effect, and therefore presumably a common structural domain with the ATPase proton pump event, and that the PS I proton pump does not insert its protons into that domain. A similar dilemma is posed from work with photosynthetic phosphorylation in chloroplasts (Ort *et al.*, 1976; Graan *et al.*, 1981) and bacteria (Melandri *et al.*, 1980; Valle-Tascon *et al.*, 1978; Baccarini-Melandri *et al.*, 1981), where the data do not fit the simple transmembrane protonmotive force as the primary energetic driving force. Those results and the data presented here cannot be explained by the existing conceptual schemes of chloroplast membrane structure and function.

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