Proton Release in Photosynthetic Water Oxidation: Evidence for Proton Movement in a Restricted Domain†

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ABSTRACT: Photosynthetic oxygen evolution of isolated spinach chloroplasts is inhibited by acetic anhydride treatment in the dark. The extent of water oxidation inhibition is correlated with an increased derivatization of membrane proteins. The chemical modifier forms stable, covalent adducts with α- and ϵ-amino groups but is reactive only with the unprotonated form of the amine. Inhibition of water oxidation occurs only when steps are taken to make the membrane leaky to protons, i.e., adding uncouplers or giving a transient exposure to 30 °C. After sensitization of the membranes to acetic anhydride inhibition in the dark, full protection of water oxidation activity can be regained by exposing chloroplasts to light, prior to addition of anhydride. The inhibition and protection against inhibition by electron-transport activity could be accounted for if there are membrane proteins involved in water oxidation, having amino functions either in the inner aqueous space or within the membrane phase. Experiments to test the two alternatives showed that the anhydride-sensitive groups are not in the inner aqueous phase. The light protection was shown to be uniquely due to proton release from photosystem II water oxidation. Proton release by photosystem I with an H^+ accumulation equivalent to that given by the protecting photosystem II activity was ineffective in bestowing protection of the water oxidation mechanism against anhydride inhibition. The data are consistent with the concept that the water oxidation apparatus releases protons initially into a sequestered domain, perhaps an intramembrane region, which cannot be reached by protons released in the photosystem I redox system. The conditions required to give anhydride inhibition of water oxidation or protection against inhibition are identical with those giving maximum derivatization or protection against derivatization of the 8-kilodalton CF_0 protein of the energy-coupling complex [Prochaska, L. J., & Dilley, R. A. (1978) *Biochem. Biophys. Res. Commun. 83*, 664–672]. Thus, the proton-releasing mechanism of the oxygen-evolving system and a part of the 8-kilodalton CF_0 component which reacts with protons released by that mechanism share a common, sequestered region, which does not interact with protons released by the photosystem I mechanism.

Photosynthetic electron-transfer reactions involve at least two proton-releasing (protolytic) steps, one being the oxidation of water by photosystem II and the other the oxidation of plastohydroquinone by photosystem I. The proton release leads to a pH gradient of near 2.7–3 units (Gaensslen & McCarty, 1971; Rottenberg et al., 1972), and the steady-state H^+ accumulation at pH 8 (external) is near 0.2–0.4 μmol of H^+/μmol of chlorophyll (Neumann & Jagendorf, 1964). Most of the protons are bound to fixed-charge buffering groups such as carboxyl functions of membrane proteins (Walz et al., 1974) either within the membrane itself or lining the inner aqueous space. In any event, the acid–base equilibria of these endogenous buffering groups ultimately includes the inner aqueous space. This fact has given support for the Mitchell view of the chemiosmotic hypothesis, namely, that the redox protolytic events *directly* acidify the inner aqueous space (Mitchell, 1966). Subsequent efflux of the accumulated protons through the CF_0–CF_1 energy-coupling complex was hypothesized by Mitchell to drive ATP[1] formation. The Williams version of chemiosmosis posits that the protolytic events release protons within the membrane, from where they directly can enter the coupling complex or indirectly equilibrate with the inner aqueous space (Williams, 1962).

If the H^+ release site of the water oxidation apparatus protrudes into the inner aqueous space, the effects of pH changes in this space, derived either from water oxidation itself or from protons released by the photosystem I protolytic event, should be indistinguishable in the way they interact with the energy-coupling apparatus or the way they could influence the oxygen-evolving system. Recent work (Giaquinta et al., 1975; Prochaska & Dilley, 1978a–c) has suggested a model wherein the H^+ releasing site of photosystem II is buried within the membrane and the protons released are "processed" to the coupling complex via a site-specific, intramembrane route. Such a model was advanced to explain data showing that photosystem II linked proton release, but not photosystem I protolytic reactions, caused conformational changes in certain membrane proteins, particularly the 8-kilodalton, DCCD-reactive hydrophobic sector of the CF_0–CF_1 energy-coupling complex (Prochaska & Dilley, 1978b,c). The deduction that it is proton release rather than some other aspect of photosystem II function came from comparative studies using proton- and electron-releasing donors (water or diphenylcarbazide) or an electron only releasing donor, iodide (Giaquinta et al., 1975; Prochaska & Dilley, 1978a; Dilley & Prochaska, 1978). Other explanations could possibly account for the data, such as conformational changes linked to an electron-transfer step, independent of protolytic reactions. This point must be tested more critically, and the results presented below deal with this question.

As background, our experiments using chemical modification reagents as probes for membrane component conforma-

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[1] Abbreviations used: Chl, chlorophyll; MV, methyl viologen; ATP, adenosine 5′-triphosphate; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; FCCP, carbonyl cyanide [*p*-(trifluoromethoxy)phenyl]hydrazone; DCMU, *N*′-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea; DCCD, dicyclohexylcarbodiimide; Ac_2O, acetic anhydride; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone.

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tional changes have shown that concomitant with the change in radioactive chemical modifier incorporation into membrane proteins between dark and light conditions, there is a change in the effect of the derivatization treatment on water oxidation activity. In general, inhibition of an enzyme increases in proportion to an increase in derivatization with chemical modifiers (Horiike & McCormack, 1979). When a light-dependent *increase* in derivatization occurs, as with the diazobenzenesulfonate reagent, water oxidation is inhibited compared to that under dark conditions (Giaquinta et al., 1975); when a light-dependent *decrease* in derivatization is observed, as with acetic anhydride (Prochaska & Dilley, 1978a), there is less inhibition of water oxidation compared to that in the dark state (such data are published in this report but were first observed by L. J. Prochaska in this laboratory). However, in the earlier work we did not test whether the light-dependent effects on water oxidation resulting from the chemical modifier treatments required just photosystem II proton release (as does the 8-kildalton CF_0 protein labeling change) or whether photosystem I alone can potentiate the effects on water oxidation activity.

Rationale for Experiments. If it can be shown that a photosystem II function is strongly affected by, say, internal pH changes which can be generated by photosystem I operating alone (DCMU present), then it is reasonable to argue that a pH-sensitive part of photosystem II protrudes into the inner aqueous space. On the other hand, if photosystem I generated internal pH changes have no effect on that particular photosystem II function but an effect *is* generated by protons released by photosystem II, then that is evidence for that functional part of photosystem II being buried within the membrane, not accessible to protons generated by photosystem I.

We can test this point by treating chloroplasts with acetic anhydride in light and dark, with both photosystems functioning or with only photosystem I (DCMU present to block photosystem II). Washing out the DCMU by two centrifugation and resuspension steps, followed by assay for H_2O oxidation activity, is a measure of the sensitivity of photosystem II to acetic anhydride inhibition under the two different treatment regimes. By analogy to the specific effects of photosystem II released protons on the 8-kilodalton CF_0 protein, we can conclude whether or not the anhydride-sensitive site in the electron-transfer Hill reaction responds to both photosystem protolytic events and thus is a site perhaps protruding into the inner aqueous space or whether there is some sort of photosystem-specific effect.

Methods

Chloroplast Isolation and Protein Assay. Chloroplast isolation, chlorophyll determination, and protein assay were as described in Prochaska & Dilley (1978a).

Electron- and Proton-Transport Assays. Electron transfer was measured by following oxygen uptake with a Clark-type electrode using methyl viologen as the electron acceptor, while pH changes of the medium were measured with pH electrodes as a means of following H^+ ion flux (Dilley, 1970). In some instances, pyocyanine was used as the cofactor for H^+ ion transport measurements. Unless otherwise specified, treatments and assays were carried out at 20 °C.

Acetic Anhydride Modification. Techniques similar to those used by Prochaska & Dilley (1978a) were followed for the acetic anhydride derivatization, except that *N*-glycylglycine was used to quench the unreacted acetic anhydride. For radioactive label experiments [^{3}H]acetic anhydride (Amersham-Searle) was used as described in the above reference.

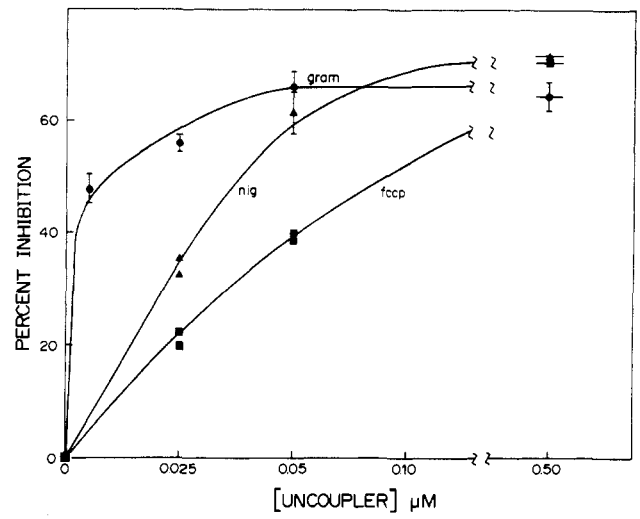

FIGURE 1: Acetic anhydride inhibits water oxidation as a function of uncoupler concentration. The reaction mixture contained in 2 mL: 100 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 50 mM Hepps–NaOH (pH 8.6), 0.5 mM methyl viologen, uncoupler [gramicidin, nigericin, or carbonyl cyanide [*p*-(trifluoromethoxy)phenyl]hydrazone (FCCP)], and chloroplasts equivalent to 40 μg of chlorophyll. Acetic anhydride at 3.5 mM was added to the chloroplast suspension in the dark for a 45-s treatment, followed immediately by addition of 50 mM *N*-glycylglycine to quench the unreacted reagent. After a total dark time of 1.5 min, the rate of electron transport ($H_2O \rightarrow$ methyl viologen) was measured. This rate was referred to an identical experiment but where the *N*-glycylglycine was added *prior* to the anhydride. Standard error bars represent the results of four separate experiments.

Details as to the composition of reaction mixtures and other conditions for each experiment are given in the figure and table legends.

Results

Evidence That Changes in Membrane Derivatization and Water Oxidation Inhibition by Acetic Anhydride Are Controlled by Local pH. Different levels of derivatization of membrane proteins with a chemical modification reagent such as acetic anhydride, primarily an amine-directed reagent, can be due to (a) changes in the availability of a functional group through changed conformational states of the membrane protein(s) or (b) changes in reactivity of a group due to, say, the degree of protonation of the NH_2 group, apart from a more primary conformational change (the protonation can result in conformational changes as a secondary effect).

The hypothesis we advanced to explain the acetic anhydride labeling data of the 8-kilodalton CF_0 component (and other membrane polypeptides; Prochaska & Dilley, 1978b) posits that *protons* released in photosystem II water oxidation exert an effect of local proticity such that protein functional groups (probably NH_2 groups of lysine residues) are less reactive with the anhydride, due to being more protonated. We recognize the need to more critically test alternatives a and b, and the experiments presented below give the results of our efforts in this direction. The experiments involve the use of two different approaches toward making membranes more leaky to protons, protonophoric uncouplers and a temperature-transition treatment, and changing the electron-transfer rate markedly while keeping the proton accumulation relatively unchanged.

Electron transfer in the Hill reaction from water to methyl viologen is resistant to acetic anhydride inhibition (30–45-s prior treatment with 3.5 mM anhydride in the dark) unless a low concentration of uncoupler is present during the dark pretreatment (Figure 1). Gramicidin, nigericin, and FCCP given at low concentrations in the dark pretreatment promote

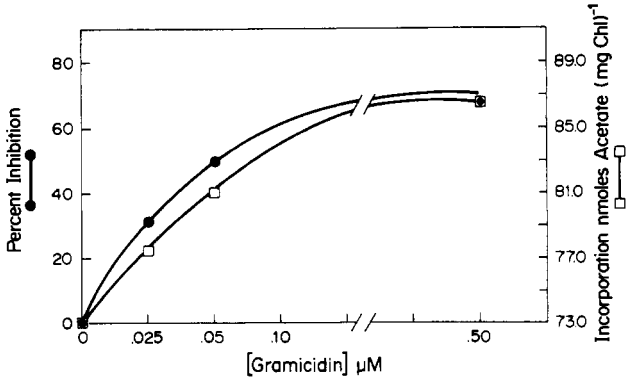

FIGURE 2: Uncoupler dependence of acetic anhydride inhibition of water oxidation and incorporation of [^{3}H]acetate from acetic anhydride. The percent inhibition curve was generated as described in Figure 1. The level of incorporation was determined by suspending chloroplasts in 5 mL of reaction medium consisting of 100 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 50 mM Hepps–NaOH (pH 8.6), 0.5 mM methyl viologen, gramicidin, and chloroplasts equivalent to 100 μg of chlorophyll. Acetic anhydride, 3.5 mM, in anhydrous methanol with [^{3}H]acetic anhydride added to give a specific activity of 5.032×10^3 cpm/nmol was added to the chloroplasts in the dark. Treatment proceeded as described in Figure 1 except samples were placed on ice following the 1.5-min incubation time. Chloroplast membranes were then centrifuged at 20000*g* for 10 min. The resulting pellet was resuspended in 2 mL of 50 mM *N*-glycylglycine (pH 8.6) and centrifuged again at 20000*g* for 10 min. This pellet was extracted in 90% aqueous acetone and centrifuged at 20000*g* for 15 min, giving a protein sediment that was finally resuspended in 0.5 mL of 5% sodium dodecyl sulfate ($NaDodSO_4$). Following a 30-min incubation at 37 °C, 0.15 mL of $NaDodSO_4$ suspension was transferred to 10 mL of Tritosol liquid scintillator. The remainder of the suspension was assayed for protein concentration by a modified Lowry method (cf. Methods).

conditions wherein acetic anhydride inhibits the Hill reaction by 60–80%. Acetic anhydride reacts with amine groups with fairly high specificity (Means & Feeney, 1971). We will assume here that only amine groups are being derivatized, but we recognize that ultimately the identity of the reactive functional group must be shown by appropriate experiments.

Figure 2 shows that the uncoupler-dependent incorporation of acetyl units from the anhydride into the membrane increases roughly in proportion to the degree of inhibition of the Hill reaction. This is consistent with the notion that the inhibition of electron transfer is related to the derivatization of anhydride-reactive groups associated with electron–proton transfer function. Because the anhydride reacts only with the uncharged (NH_2) form of amine groups, and given the inhibition-promoting effects of three quite different uncouplers, we interpret these data as evidence that both the anhydride reactivity of certain membrane amine functions and the anhydride inhibition of the Hill reaction must be sensitive to pH conditions in some membrane region not readily accessible to the external phase (in times <1 min at least). As will be shown below, the membrane region in question is not identifiable with the inner aqueous phase. The above uncoupler effects are obtained in dark-held chloroplasts, thus, there seems to be a "pool" of protons retained in the membrane in a metastable state. Apparently, the uncouplers allow this putative, local acidic phase to equilibrate with the pH 8.6 conditions of the suspending medium, leading to pH conditions alkaline enough to shift the amine groups to the NH_2, or reactive form.

Measurement of pH changes of chloroplasts suspended in conditions similar to those of Figure 1, except with 0.5 mM Hepps buffer, with no methyl viologen, and with either 40 or 60 μg of Chl/mL, showed that addition of gramicidin, in the dark, resulted in efflux of protons. With the pH-measuring apparatus capable of detecting a change of 1 nmol of H^+/mg of Chl, a chloroplast suspension initially at pH 8.6, with 40 μg of Chl/mL gave an efflux of 35 ± 6 nmol of H^+/mg of Chl when 0.25 μM gramicidin was added. Subsequent additions of gramicidin did not cause a significant pH change. In another experiment with 60 μg of Chl/mL and the initial pH at 8.3, the first addition of 0.1 μM gramicidin resulted in a proton efflux of 35 ± 1 nmol of H^+/mg of Chl, with the second gramicidin addition having essentially no effect. The conditions of dim background room light, temperature, and chloroplast treatment prior to the pH measurements were similar to the conditions used for the acetic anhydride derivatization and electron-transfer inhibition experiments.

It is unlikely that the three unrelated uncouplers would have a common effect on membrane derivatization and water oxidation, except through their effects as protonophores. However, to test this point, we employed a temperature-shift approach to increase proton permeability. Proton permeability increases markedly with increasing temperature, the Q_{10} being 1.4 (Neumann & Jagendorf, 1964); but more to the point, Takahama et al. (1977) have shown that a temperature jump induces a rapid efflux of protons from dark-held, but preilluminated, thylakoids. We used a slower temperature transition, going from 20 to 30 °C in a span of 30 s and then back down to 20 °C in another 20-s period, followed by acetic anhydride treatment (all this in the dark) and subsequent assay for Hill reaction activity ($H_2O \rightarrow$ methyl viologen) and membrane derivatization. Table I shows that the temperature transition converted the thylakoids from being resistent to Hill reaction inhibition by the anhydride (in the absence of uncoupler) to being sensitive. A brief light exposure given to the temperature-treated, sensitive thylakoids returned them to a state of being more resistent to anhydride. The derivatization pattern observed is consistent with the inhibition; i.e., a greater level of acetylation was found when the Hill reaction was more inhibited. Neither the protection against water oxidation inhibition nor the level of derivatization returned exactly to the control level after the light exposure, but the trend clearly is evident. The data are consistent with the notion that a metastable "proton pool" seems to be present, in the dark, in the thylakoid system. Although the chloroplasts were kept in stringent dark conditions during the dark phase of the experiments, they had been exposed to room light during the isolation. Other workers have also noted what appears to be an acidic condition within the thylakoid system that is retained in dark conditions (Takahama et al., 1977; Enser & Heber, 1980; Joliot & Joliot 1980).

A test of whether the observed light-dependent protection against acetic anhydride inhibition may be better correlated to an electron-transfer step rather than to proton involvement was carried out by varying electron-transfer rate by light intensity at two different uncoupler concentrations and by varying uncoupler concentrations at constant light intensity. Table II shows that the rate of electron flow does not correlate well with protection against anhydride inhibition. Parts A and B of Table II, where the electron-transfer rate was varied by light intensity, show that while higher electron-transfer rates lead to more protection, there is no close correlation to the rate of the redox reaction, rather the controlling factor appears to be the uncoupler concentration. At the lower gramicidin level (part A), a low electron-transfer rate [170 μequiv h^{-1} (mg of Chl)$^{-1}$] with medium light intensity permitted only 17% inhibition. At the higher gramicidin level (part B), medium light intensity gave a much higher rate of electron flow [280 μequiv h^{-1} (mg of Chl)$^{-1}$] but gave *less* protection against anhydride inhibition. If the protected state were due to conformational

Table I: Temperature Transition Effect on Electron Transport Activity and Acetic Anhydride Derivatization of Thylakoids[a]

treatment	% inhibition of $H_2O \rightarrow MV$ act. [μequiv h^{-1} (mg of Chl)$^{-1}$]	incorporation of acetyl units from [^{3}H]Ac_2O [nmol of acetyl (mg of protein)$^{-1}$]
(A) control, no T-jump		
(1) −uncoupler	0	69.0
(2) +0.05 μM gramicidin	86	92.3
(B) T-jump		
(1) dark conditions, no uncoupler added initially	58	90.1 ± 0.3
(2) light given after the T-jump, then dark treatment with anhydride, no uncoupler added initially	20	85 ± 2

[a] (A) Chloroplasts (20 μg of chlorophyll/mL) were suspended in a medium at 20 °C containing 50 mM Hepps–NaOH (pH 8.6), 100 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM methyl viologen, and, where indicated, 0.05 μM gramicidin. Treatment with acetic anhydride at 3.5 mM was for 45 s followed immediately by the addition of 50 mM *N*-glycylglycine as a quench. Stringent dark conditions were maintained throughout the treatment. For determination of percent inhibition, water to methyl viologen Hill activity was then assayed with saturating light and referenced to an identical experiment in which the *N*-glycylglycine was added to the anhydride. As in the Figure 3 experiment gramicidin was added to a final concentration of 5 μM before the final electron-transport assay to assure that none of the treatment effects were due to different uncoupling levels. In the case where the anhydride was added first, the final electron-transport rate in the absence of uncoupler was 432 μequiv h^{-1} (mg of Chl)$^{-1}$, and with uncoupler (0.05 μM, added initially) present, it was 102 μequiv h^{-1} (mg of Chl)$^{-1}$. (B) Chloroplasts (20 μequiv of chlorophyll/mL) were incubated in stringent dark conditions for 30 s in a medium at 30 °C containing 50 mM Hepps–NaOH (pH 8.6), 100 mM sucrose, 50 mM KCl, and 2 mM $MgCl_2$. Following this treatment, the suspension was cooled to 20 °C in ~20 s, at which time 0.5 mM methyl viologen was added. (1) An aliquot of those chloroplasts was then treated, in the dark, with 3.5 mM acetic anhydride for 45 s, followed immediately by the addition of 50 mM *N*-glycylglycine. Percent inhibition of water to methyl viologen Hill activity was then determined as described in (A). (2) Another aliquot of the 30 °C treated chloroplasts was given a 15-s saturating light exposure, followed by a 45-s dark period. At this time, 3.5 mM acetic anhydride was added, and after 45 s, unreacted anhydride was quenched by the addition of 50 mM *N*-glycylglycine. Percent inhibition of water to methyl viologen Hill activity was then assayed as described in (A). Incorporation of [^{3}H]acetic anhydride into the membrane was determined in all of the above cases by the procedure described in Figure 2.

Table II: Effect of Light Intensity on Uncoupler-Mediated Acetic Anhydride Inhibition of Water → Methyl Viologen Hill Activity[a]

	light intensity	electron transport rate [μequiv h^{-1} (mg of Chl)$^{-1}$]	% inhibition of $H_2O \rightarrow MV$
(A) 0.025 μM gramicidin	0	0	57
	medium	170 (47)	17
	high	360 (96)	0
(B) 0.50 μM gramicidin	0	0	74
	low	110	64
	medium	280	33
	high	385	0
(C) 0.025 μM nigericin and 0.025 μM valinomycin	high	237	6
0.5 μM nigericin and 0.5 μM valinomycin	high	465	56

[a] Chloroplasts equivalent to 20 μg of chlorophyll/mL were suspended in a medium containing 50 mM Hepps–NaOH (pH 8.6), 100 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM methyl viologen, and the indicated concentration of either gramicidin or nigericin and valinomycin. Electron-transport rates were then assayed under low, medium, or high light conditions. Acetic anhydride at 3.5 mM was added in the light, and after 45 s, the unreacted anhydride was quenched by the addition of 50 mM *N*-glycylglycine. Electron-transfer activity was allowed to continue for an additional 45 s before turning off the light. After a 2-min dark phase, electron-transport activity was again assayed in saturating light with 5 μM gramicidin added to ensure that the rates were maximally uncoupled. For determination of the percent inhibition, this final rate was referenced to an identical experiment, except that the *N*-glycylglycine was added prior to the anhydride. The numbers in parentheses are the extents of the steady-state H^+ accumulation in nanomoles per milligram of chlorophyll.

changes in membrane proteins linked to electronic, rather than protonic, effects, then the latter conditions should have resulted in a lesser inhibition, not the greater inhibition observed.

Part C, Table II, shows the effect of keeping the light intensity constant and varying the potent uncoupling combination of nigericin plus valinomycin. Again, it is evident that high electron transport rates do not correspond to increased protection, but to just the opposite; hence, we interpret this as evidence that the higher uncoupler concentration makes the membrane leaky enough to protons to dissipate a proton gradient faster than the protolytic reactions can supply protons, allowing the anhydride-sensitive state to occur.

Demonstration That Electron-Transfer Conditions Protect against Acetic Anhydride Inhibition of the Hill Reaction. The "phase" referred to above, the acidity of which apparently controls the extent of anhydride reaction with membrane components and inhibition of the Hill reaction, responds to electron transport, even in the presence of low concentrations of uncouplers, so as to have the less reactive state restored. The experiment to show this point consists of treating chloroplasts with either light or dark conditions during the 45-s exposure to acetic anhydride followed by recording oxygen uptake in the methyl viologen Hill reaction (Figure 3). Figure 3A shows the inhibitory effect of a dark treatment with 3.5 mM acetic anhydride and 0.05 μM gramicidin. The relevant rate to consider is the final rate, after addition of *N*-glycylglycine to quench the unreacted anhydride and additional gramicidin to assure that full uncoupling is obtained in all the final assay conditions. The dark condition rate of 193 μequiv h^{-1} (mg of Chl)$^{-1}$ can be compared to the rate of 502 μequiv h^{-1} (mg of Chl)$^{-1}$ obtained when a similar chloroplast suspension was illuminated during the anhydride treatment (Figure 3B). The conditions of illumination clearly protect against the anhydride inhibition of the Hill reaction. This is not a general light effect, e.g., a destruction of the anhydride, in that activation of photosystem II is specifically required to obtain the protection. Certain controls will be considered before those experiments are presented.

The effectiveness of the *N*-glycylglycine quench is seen in the data of Figure 3C,D. The quench reagent, added before the acetic anhydride, protects against the dark-state inhibition otherwise found when the anhydride is added before the *N*-glycylglycine (Figure 3C). When the quench reagent is added in the light before the acetic anhydride (Figure 3D), the net effect is similar to the case where acetic anhydride is added before the quench reagent in the light (Figure 3B).

Site of Acetic Anhydride Inhibition of Electron Transport. Partial reactions associated with either photosystem I or II were measured after acetic anhydride treatment to find the inhibitory site. Chloroplasts were treated with 3.5 mM acetic anhydride (plus 0.05 μM gramicidin) in the dark to elicit the usual 70% inhibition of $H_2O \rightarrow MV$ activity. The photosystem II partial reaction, $H_2O \rightarrow$ silicomolybdate (+DCMU)

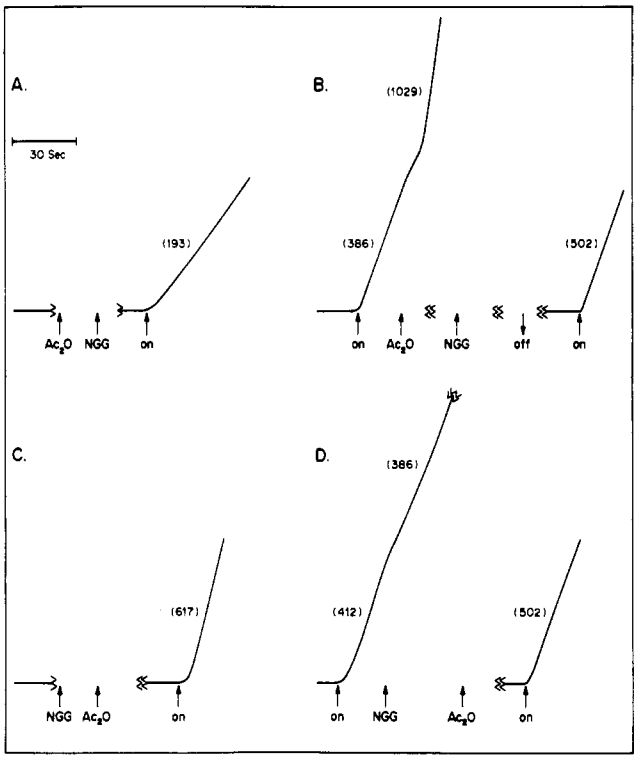

FIGURE 3: Protection against acetic anhydride (Ac_2O) inhibition of water oxidation by light-induced electron flow. Gramicidin at 0.05 μM was present initially in all the reactions; other conditions were as described in Figure 2. Just before turning on the light for the final assay of the rate, 5 μM gramicidin was added to assure that complete uncoupling occurred in all cases. (A) This shows dark inhibition [compare to (B)]. Dark inhibition levels were determined as described in Figure 2 with the electron-transport rate being indicated by the number in parenthesis. (B) This shows light protection, but the light was turned on before Ac_2O was added. Ac_2O was present for 45 s, NGG was added, and after an additional 45 s the light was turned off. A 2-min dark incubation was then given before the electron-transport rate ($H_2O \rightarrow$ methyl viologen) was measured. (C and D) Controls performed under identical time regimes as the traces immediately above in the figure, showing that *N*-glycylglycine is an effective quencher of the Ac_2O whether added in dark (C) or light (D) conditions prior to the Ac_2O.

(Giaquinta et al., 1975) was measured in control and anhydride-treated samples, with the results shown in Figure 4A,B. It is clear that acetic anhydride severely inhibits this partial reaction (a control rate of 230 μequiv of e^- h^{-1} (mg of Chl)$^{-1}$ vs. 15 for the anhydride-treated sample). In those anhydride-treated chloroplasts, but without DCMU present and with the addition of I^- as a donor to photosystem II and methyl viologen as an acceptor, there was a restoration of electron transport to 78 μequiv of e^- h^{-1} (mg of Chl)$^{-1}$, (Figure 4C) suggesting that the water oxidation mechanism was the principal target of anhydride inhibition. In the $I^- \rightarrow$ MV reaction, addition of 5 μM DCMU inhibited the rate down to 16 μequiv of e^- h^{-1} (mg of Chl)$^{-1}$. A proton-donating photosystem II donor, diphenylcarbazide, also showed donor activity with chloroplasts having the water oxidation activity inhibited by acetic anhydride. Chloroplasts in which the anhydride gave a 75% inhibition of water oxidation [992 to 244 μequiv h^{-1} (mg of Chl)$^{-1}$] showed a restoration of electron transport by diphenylcarbazide (DCMU sensitive) back to 393 μequiv h^{-1} (mg of Chl)$^{-1}$. Thus there was no indication that the e^- only donor (I^-) differed much from the e^-–H^+ donor in restoring photosystem II electron transport in anhydride-inhibited chloroplasts.

Because the silicomolybdate reduction is a complex situation, we also tested for the effect of acetic anhydride on the reduction of silicomolybdate when DCMU was not present. Figure 4D, left side, shows that the anhydride inhibits that reaction also. Hence, there is no likelihood that the inhibition seen in Figure 4B is due to a selective acetic anhydride inhibition of the electron feed-out to silicomolybdate which occurs in the presence of DCMU. Leonard et al. (1978) have shown that an extractable "factor" is present in chloroplasts which is necessary for optimum rates of silicomolybdate reduction with DCMU present.

In another experiment we added acetic anhydride to a $H_2O \rightarrow$ silicomolybdate + DCMU reaction *in the light* and observed no inhibition (data not shown). This is similar to the pattern of protection, in the light, found for $H_2O \rightarrow$ methyl viologen (Figure 3B).

A photosystem I partial reaction, durohydroquinone (DQH_2) $\rightarrow$ MV (+DCMU) (White et al., 1977; Izawa & Pan 1978) was found to be unaffected by 3.5 mM acetic anhydride (Figure 5). Thus, the anhydride inhibitory site(s) is associated with redox events of photosystem II, particularly water oxidation. A second, less sensitive, inhibitory site between the site of I^- donation and plastoquinone reduction was indicated by the low level of $I^- \rightarrow$ MV activity noted in the data of Figure 4C.

The next experiments involved testing whether the protolytic and/or electron-transfer steps of either photosystem I or II were equally effective in protecting against the anhydride inhibition of the photosystem II functions shown above.

Photosystem Specificity for Protection of Electron-Transfer Inhibition by Acetic Anhydride. As mentioned in the introduction, the results of our studies of membrane labeling with the three chemical modifiers indicated that the light-dependent derivatization pattern was totally dependent on proton release by either water oxidation or an alternative photosystem II proton-releasing donor such as diphenylcarbazide (Prochaska & Dilley, 1978a). The uncoupler insensitivity of the light–dark effect on membrane labeling and the dependence on proton release in a photosystem II oxidation are consistent with the hypothesis that the protons released in the photosystem II oxidation are initially restricted to a specific "domain" during the time required to elicit the change in derivatization (15–45 s). The question now posed is whether the protection against anhydride inhibition of water oxidation is similarly dependent specifically on photosystem II linked protons or whether photosystem I generated protons can confer protection. If the inhibitor site in water oxidation is exposed to the inner aqueous space, then photosystem I generated protons, by reaching the inner aqueous space, could protonate putative lysine amine groups, making them unreactive with acetic anhydride, thus protecting against derivatization of the functional group(s) and subsequent inhibition of water oxidation. On the other hand, we can propose a scheme wherein the sensitive site in water oxidation is sequestered within the membrane, unavailable to photosystem I generated protons.

To test these alternatives, we had to devise a more elaborate protocol than that used to demonstrate photosystem selectivity in controlling the derivatization of the 8-kilodalton CF_0 component (Prochaska & Dilley, 1978b,c). Namely, we had to be able to treat the chloroplasts with acetic anhydride under conditions where only photosystem I electron and proton transfer occurs (i.e., plus DCMU), compared to when photosystem II also functions; but it was also necessary to carry out an assay for water oxidation activity after the treatment and subsequent removal of DCMU. This was accomplished by two centrifugation–resuspension steps, so as to remove most of the DCMU. Table III shows the results of such an ex-

Table III: Photosystem I Does Not Protect Water Oxidation against Acetic Anhydride Inhibition[a]

treatment conditions	Ac_2O (mM)	Hill reaction ($H_2O \rightarrow$ MV) act. remaining after Ac_2O treatment [μequiv of $e^- h^{-1}$ (mg of Chl)$^{-1}$] 0.1 μM FCCP	0.01 μM gramicidin
(1) dark + DCMU	3.5	245	150
(2) light, photosystem I only, + DCMU + pyocyanine + reductant	3.5	247	181
(3) light, photosystem I + II, + pyocyanine + reductant	3.5	546	464

[a] Chloroplasts were suspended in 40 mL of reaction medium containing 100 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 50 mM Hepps-NaOH (pH 8.6), 30 μM pyocyanine, 1 mM dithioerythritol, 1.2 μM DCMU where indicated, 0.8 mg Chl, 0.10 μM FCCP or 0.01 μM gramicidin, and 3.5 mM acetic anhydride. The treatment time was 45 s, followed immediately by the addition of 50 mM *N*-glycylglycine to quench the unreacted anhydride. Treatments were as follows. (1) dark, DCMU, and acetic anhydride; conditions giving inhibition of water oxidation. The treated suspension was centrifuged at 12000*g* for 1 min, resuspended in 35 mL of 100 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, and 50 mM Hepps-NaOH (pH 8.6), centrifuged again, and finally resuspended in 0.3 mL of that medium. (2) Photosystem I light conditions. Acetic anhydride was added 30 s after the light was turned on. Following treatment, chloroplasts were processed as described in (1). (3) Photosystem II + I light conditions. Acetic anhydride was added 30 s after the light was turned on. The resuspended samples (1-3) were then assayed for $H_2O \rightarrow$ methyl viologen electron-transfer activity at 20 μg of Chl/mL with 5 μM gramicidin added to assure that all samples were uncoupled. Light intensities for flasks 2 and 3 were adjusted at each uncoupler concentration to give the same net proton accumulation of ~70 nmol of H^+ (mg of Chl)$^{-1}$. Under conditions for photosystem II + I activity, 100% light protection against anhydride inhibition of H_2O oxidation was observed at 0.10 μM FCCP and 0.01 μM gramicidin. The higher light intensity used to drive photosystem I (+DCMU) activity similarly demonstrated full light protection at 0.1 μM FCCP and 0.01 μM gramicidin in a prior $H_2O \rightarrow$ methyl viologen assay. These measures of light protection were determined according to the procedure given in the legend of Figure 3.

periment. To be meaningful, it was necessary to adjust the conditions so as to have equivalent extents of H^+ uptake generated in the photosystem I alone case and the photosystem II plus I case. This was accomplished by adjusting the light intensity, so that the extent of H^+ uptake was ~50 nmol of H^+ (mg of Chl)$^{-1}$ for both cases. Obviously, the amount of uncoupler added had to be high enough to elicit dark inhibition by acetic anhydride (cf. Figure 1) but not so high as to abolish the H^+ uptake.

Table III shows that low concentrations of gramicidin (0.01 μM) or FCCP (0.1 μM) elicit dark inhibition of the $H_2O \rightarrow$ methyl viologen Hill reaction and photosystem I activity in the light does not protect against such inhibition (line 1 compared to 2). However, photosystem II plus photosystem I activity protects against the anhydride inhibition (line 3). During these experiments, we noted that higher uncoupler levels permitted photosystem I activity to bestow a partial protection against anhydride inhibition, as shown in Table IV. At the highest gramicidin and FCCP concentrations shown (0.05 and 0.5 μM, respectfully), photosystem I only conditions resulted in a final Hill reaction rate of 145 vs. 77 μequiv of $e^- h^{-1}$ (mg of Chl)$^{-1}$ for the dark-inhibited state (plus gramicidin) and 209 vs. 28 μequiv of $e^- h^{-1}$ (mg of Chl)$^{-1}$ for FCCP. Nigericin at 0.05 μM showed a comparable level of partial protection when photosystem I only conditions prevailed. In all three cases photosystem II plus photosystem I light conditions gave considerably more protection against anhydride inhibition (line 5, Table IV).

Acetic Anhydride Labeling of the 8-Kilodalton CF_0 Protein. Details [beyond those reported by Prochaska & Dilley (1978b)] of the acetic anhydride labeling of the 8-kilodalton CF_0 protein will be published separately, but it should be pointed out here that a close correspondence occurs between the uncoupler-induced increased labeling of the thylakoid membrane (Figure 1) and the labeling of 8-kilodalton CF_0 protein. For example, when the whole membrane labeling in the dark increased from 59 nmol of acetyl bound/mg of protein (minus uncoupler) to 83 nmol for the case with 0.05 μM gramicidin; the labeling of 8-kilodalton CF_0 protein increased

Table IV: Effect of Higher Uncoupler Concentrations on Photosystem I and Photosystem I + Photosystem II Protection of Water Oxidation Inhibition by Acetic Anhydride[a]

treatment conditions	Ac_2O (mM)	Hill reaction ($H_2O \rightarrow$ MV) act. remaining after Ac_2O treatment [μequiv of $e^- h^{-1}$ (mg of Chl)$^{-1}$] 0.05 μM gramicidin	0.05 μM nigericin	0.5 μM FCCP
(1) dark	0	463	341	283
(2) dark + DCMU	0	347[b]	275[b]	246[b]
(3) dark + DCMU	3.5	77	62	28
(4) light, photosystem I only, + DCMU + pyocyanine + reductant	3.5	116 (145)[c]	137 (164)[c]	185 (209)[c]
(5) light, photosystem I + II, + pyocyanine + reductant	3.5	257	344	332

[a] Chloroplasts equivalent to 800 μg of chlorophyll were suspended in 40 mL of reaction medium containing 100 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 50 mM Hepps-NaOH (pH 8.6), 30 μM pyocyanine, 1 mM dithioerythritol, 1.25 μM DCMU where indicated, 0.05 μM FCCP, and 3.5 mM acetic anhydride where indicated. The treatment time was 45 s, followed immediately by the addition of 50 mM *N*-glycylglycine. Treatments were as follows. (1) dark control. Chloroplasts were centrifuged at 12000*g* for 1 min, resuspended in 20 mL of 200 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 0.5 mg of BSA/mL, and 5 mM Hepes-NaOH (pH 7.5), centrifuged again, and finally resuspended in 0.3 mL of that medium. All other treatments were given similar centrifugation and resuspension steps. (2) Dark, DCMU, and acetic anhydride; a test of the residual DCMU carried over into the final assay. (3) Dark, DCMU, and acetic anhydride; conditions giving the inhibition of water oxidation. (4) Photosystem I light conditions. The acetic anhydride was added 30 s after the light was turned on. (5) Photosystem II + I light conditions. The acetic anhydride was added 30 s after the light was turned on. The resuspended samples (1-5) were then assayed for $H_2O \rightarrow$ methyl viologen electron-transfer activity at 20 μg of Chl/mL with 5 μM gramicidin added to assure that all samples were uncoupled. Light intensities for flasks 4 and 5 were adjusted to give the same net H^+ accumulation of ~50 nmol of H^+ (mg of Chl)$^{-1}$. Under conditions of photosystem II + I activity, 97% light protection against anhydride inhibition of water oxidation was observed at 0.05 μM gramicidin, 80% at 0.05 μM nigericin, and 97% at 0.5 μM FCCP. The higher light intensity used to drive photosystem I (+DCMU) activity similarly demonstrated 100% light protection at 0.05 μM gramicidin, 83% at 0.05 μM nigericin, and 100% at 0.5 μM FCCP in a prior $H_2O \rightarrow$ methyl viologen assay. These measures of light protection were determined according to the procedure given in the legend of Figure 3. [b] The effect of residual DCMU remaining after washing and resuspension. [c] This represents the estimated rate had DCMU *not* been present (cf. treatment 1 vs. treatment 2).

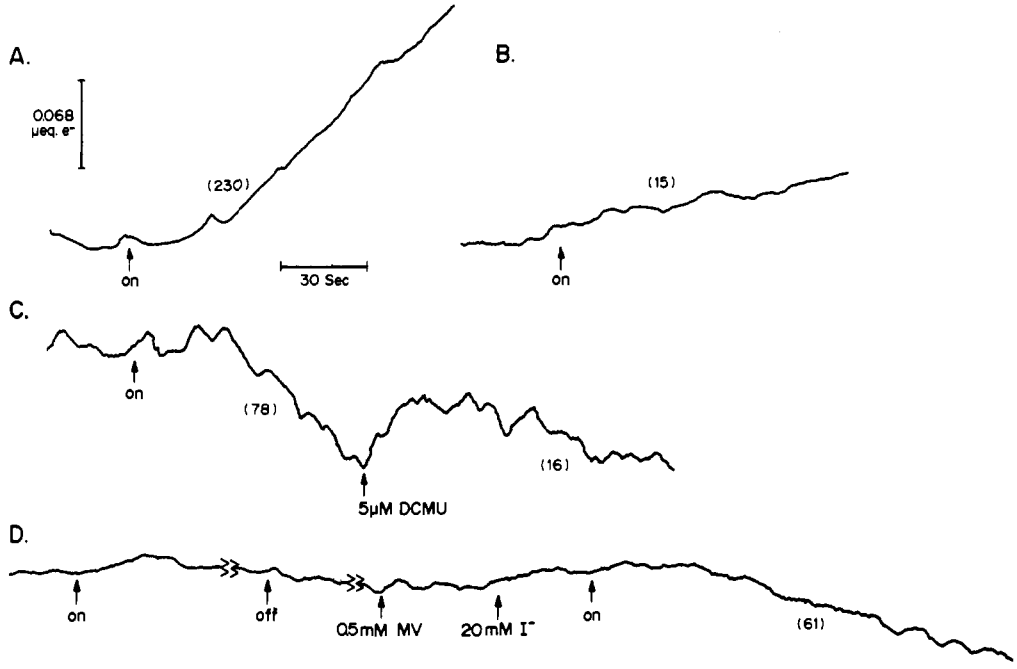

FIGURE 4: Acetic anhydride inhibits water oxidation but not electron transport with iodine as an alternate photosystem II donor. (A) Chloroplasts equivalent to 800 μg of chlorophyll were suspended in 40 mL of reaction mixture consisting of 100 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 50 mM Hepps–NaOH (pH 8.6), 1.2 μM DCMU, and 0.05 μM gramicidin. *N*-Glycylglycine, 50 mM, was added to the chloroplast suspension in the dark for a 45-s treatment, followed immediately by addition of 3.5 mM acetic anhydride. An upward deflection of the trace represents O_2 evolution. (B) This reaction mixture was treated identically with (A) except the anhydride was added prior to the *N*-glycylglycine. Following treatment, (A) and (B) were each diluted with 40 mL of reaction mixture containing 100 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, and 50 mM Hepes–NaOH (pH 7.5). These were centrifuged at 4000*g* for 6 min to give a pellet that was resuspended in 0.6 mL of the same medium. Treated chloroplasts equivalent to 40 μg of chlorophyll were added to 2 mL of that medium, followed by the addition of 1.2 μM DCMU and 100 μL of glycerol. Silicomolybdate, 140 μM, was added just prior to saturating light exposure, and the rate of electron flow ($H_2O \rightarrow$ silicomolybdate) was measured, the rate being indicated by the number in parentheses. (C) To chloroplasts that were treated as in (B) (but without addition of DCMU or silicomolybdate) to elicite the inhibition of H_2O oxidation, were added 0.5 mM methyl viologen and 20 mM potassium iodide. After a dark incubation of 45 s the rate of electron flow ($I^- \rightarrow$ MV) was measured and was found to be sensitive to 5 μM DCMU. This shows that the acetic anhydride inhibition seen in (B) was primarily at the level of water oxidation. (D) Treatment B was repeated except that no DCMU was present during the $H_2O \rightarrow$ silicomolybdate electron-transport assay. This shows that $H_2O \rightarrow$ silicomolybdate electron flow is inhibited also when DCMU is absent [the acceptor can accept electrons from near the plastoquinone pool (Giaquinta & Dilley 1975)]. The calibration bar refers to (A), (B), and (D); for (C) the sensitivity was 2.5-fold greater.

from 0.70 to 0.96 nmol of acetyl/nmol of 8-kilodalton protein. The conditions of reaction were similar to those given for Figure 2. The 8-kilodalton protein was isolated by the Nelson et al. (1977) procedure and purified on $NaDodSO_4$–polyacrylamide gel electrophoresis gels, and the radioactivity was measured in the 8-kilodalton band. Photosystem II water oxidation activity, but not photosystem I activity, keeps the level of labeling of the 8-kilodalton CF_0 protein at a lower level than that observed in the dark (Prochaska & Dilley, 1978b). Typical data for the conditions similar to those of Figure 2 with 0.05 μM gramicidin present is as follows: dark, 0.96 nmol of acetyl/nmol of 8-kilodalton CF_0 protein; light ($H_2O \rightarrow$ methyl viologen), 0.77 nmol of acetyl/nmol of protein.

Discussion

Electron Transfer Inhibition by Acetic Anhydride. Acetic anhydride reacts with chloroplast membrane proteins, probably acetylating lysine ϵ-NH_2 groups or α-NH_2 groups of *N*-terminal amino acid residues, reacting only when the amine function is unprotonated (Means & Feeney, 1971). Acetylation of chloroplast membranes leads to inhibition of water oxidation activity (Figures 1 and 4), but, interestingly, the inhibition requires a pretreatment with one of several diverse uncouplers (Figure 1), or a temperature transition (Table I)—treatments which are known to cause increased proton permeability. Such uncoupler and temperature effects are most simply interpreted as due to the treatment releasing protons from a space existing at a pH lower than the externally buffered (pH 8.6) phase. Upon equilibration with the pH 8.6 conditions, NH_3^+ groups might deprotonate to the reactive $\ddot{N}H_2$ state, leading to greater derivatization and subsequent inhibition of certain functions.

Direct observation of gramicidin-induced proton efflux (in the dark) from chloroplast membranes showed that ~35 nmol of H^+/mg of Chl are lost to the medium when the suspension medium is at pH 8.3–8.6 (See Results). This correlates quite well with the increased derivatization induced by the addition of uncoupler in the dark; those values range from 15 to 45 nmol of acetyl/mg of Chl, the results varying with different chloroplast preparations. A calculation shows that 35 nmol of H^+/mg of Chl cannot be accounted for only by free protons in the inner aqueous space (2×10^{-4} nmol H^+/mg Chl assuming a volume of 20 μL/mg of Chl), so nearly all the protons are being released from buffering groups such as amines. From the known buffer capacity of chloroplasts at pH values near 8.3–8.6 (Waltz et al., 1974), it can be seen that a pH shift of ~0.2 pH unit could account for the release of 35 nmol of H^+/mg of Chl. This calculation assumes that half the buffer capacity measured at pH 8.6 is located inside the membrane.

The alternative that the uncoupler and heating effects involve some sort of rearrangement of membrane components, apart from pH effects, leading to a greater accessibility of amine functional groups is not absolutely ruled out, but it seems a very unlikely explanation. The light-dependent protection of the Hill reaction activity and the decreased labeling of the membrane proteins cannot be explained as due to a direct link to an electron-transfer step, because, as shown in

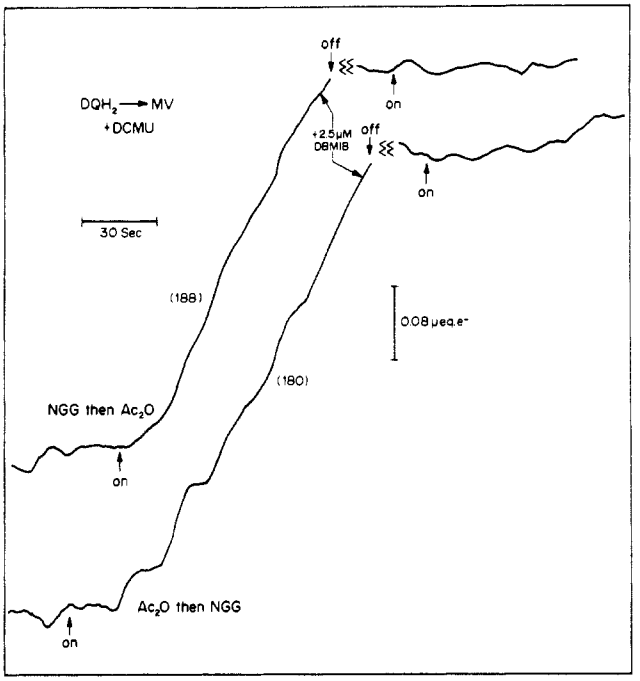

FIGURE 5: Acetic anhydride has no effect on the partial photosystem I reaction, durohydroquinone → methyl viologen. Treatment conditions were as described for Figure 5A,B, except that the final suspension of chlorophyll included the addition of 5 μM DCMU, 2.5 μM gramicidin, 0.5 mM methyl viologen, and 62.5 μM durohydroquinone. The measured electron-transport rate in saturating light is indicated in parentheses. In each case, the rate is shown to be completely inhibited by the addition of 2.5 μM DBMIB.

Table II, faster electron transfer occurred in some treatments where there is an increase in labeling and increased inhibition of the Hill reaction. Moreover, at sufficiently high uncoupler concentrations there is a reversal of the light protection (Table II, part c), which could be due to the uncoupler mediating a rapid enough proton loss from the thylakoid to permit the formation of the $\ddot{N}H_2$ form of amine groups associated with water oxidation.

That the anhydride-sensitive inhibition site(s) are associated primarily with water oxiddation and to a lesser extent with a second site between the photosystem II donation site and plastoquione is indicated by studies of partial reactions. A proton-donating photosystem II donor, diphenylcarbazide (see Results), or the electron-only donor, I^- (Figure 4), both restored photosystem II dependent electron transfer in chloroplasts previously inhibited by acetic anhydride treatment. A photosystem I donor reaction, durohydroquinone → methyl viologen, was not affected (Figure 5), whereas H_2O → silicomolybdate + DCMU was severly inhibited by acetic anhydride (Figure 4). Iodine (as a photosystem II donor) to methyl viologen electron-transfer activity, where the I^- substitutes for the anhydride-inhibited H_2O oxidation, was only partially inhibited (Figure 4), thus identifying the second, less sensitive inhibition site.

Light Protection against Acetic Anhydride Inhibition: Photosystem II Specific Effects. The acetylation of certain protein functional groups and the resultant inhibition of electron transfer can be protected against by allowing light-dependent water oxidation to occur while the chloroplasts are exposed to the acetic anhydride (Figure 3 and Table II). We interpret the protection against water oxidation inhibition as due to protonation of protein ϵ- and α-NH_2 groups by protons released by the protolytic redox reaction(s). It is clear from Tables III and IV that water oxidation conditions (proton release) were far more effective than photosystem I conditions in providing protection. This is the key point which argues for a restricted space or an intramembrane domain which contains the anhydride-sensitive target groups of the water oxidation system. We propose a model wherein the water oxidation mechanism releases protons directly into this sequestered domain, leading to protonation of amine groups and protection of the water oxidation system against anhydride inhibition. It follows that if the protected state is due to protonated amine groups and if those groups were in the inner aqueous space, then *either* photosystem protolytic event at equal H^+ accumulation levels should have provided protection since it is believed that protons released by both photosystems ultimately acidify the inner aqueous space (Kraayenhof et al., 1972). However, because only photosystem II generated conditions (protons) were effective in eliciting protection (Tables III and IV) when a low concentration of uncoupler is present, we can conclude that one way of explaining this is by proposing that normally the "domain" in question cannot be reached by protons contained within the inner aqueous space. Consistent with this is the finding that simply by raising the concentration of uncoupler, photosystem I generated protons become somewhat effective in bestowing protection on the Hill reaction (compare Table IV with Table III). This fact is quite consistent with the model we are suggesting, and it is strong evidence favoring protonation levels in a membrane subcompartment as a critical factor determining the anhydride reactivity and inhibition of water oxidation. If uncouplers are necessary to facilitate acetic anhydride inhibition in the dark (by allowing protons to leak out of a region), then the uncoupler could aid in allowing the protons, accumulated in the inner aqueous space by photosystem I, to "reach" the putative intramembrane, normally photosystem II specific domain. The uncoupler effectiveness is rather weak in permitting photosystem I linked protons' access to the amine group(s) involved in the anhydride inhibition compared to protection bestowed by photosystem II activity.

Following the original suggestion by Mitchell (1966), it has been almost a "central dogma" of workers in this research area that water oxidation and oxidation of the plastohydroquinone directly release protons into the inner aqueous space. Certain data have been consistent with this view, such as the Bowes & Crofts (1978) delayed-light experiments and the Junge et al. (1978) neutral red kinetic experiments. Further work is necessary to resolve the issue, for it is not easy to reconcile the present results with those of the above-mentioned workers.

Water Oxidation Proton Release and the 8-Kilodalton CF_0 Protein. The relationship of water oxidation proton release with protection of photosystem II against anhydride inhibition is virtually identical with the regulation of [^{3}H]acetic anhydride labeling of the 8-kilodalton CF_0 protein (Prochaska & Dilley, 1978b,c). In both cases the evidence suggests that $\ddot{N}H_2$ groups are driven to the anhydride-unreactive NH_3^+ form by protons released in photosystem II oxidations, photosystem I redox–protolytic activity being unable to mimic the effects.

In the proposed model both the anhydride-sensitive site(s) associated with water oxidation and the anhydride-reactive site on the 8-kilodalton CF_0 protein share a common, restricted domain. This domain may be an intramembrane region defined by certain membrane proteins which could provide either structured water and/or amino acid side groups for a pathway specifically connecting the proton-release site(s) in water oxidation with the CF_0 protein (Nagle & Morowitz, 1978). An alternative might be some sort of membrane surface or interfacial phenomenon that provides a sequestered region on the inner boundary of the thylakoid (Kell, 1979), although it

seems more difficult with this model to explain the failure of photosystem I linked protons to mimic the photosystem II effects. As an aside, it is probably not reasonable to expect the photosystem specificity to be accounted for by kinetic arguments, because the membrane labeling and inhibition pattern occurs over a 30–45-s time frame, enough time to assume that steady-state conditions prevail.

Protons which reach the CF_0 channel or "proton well" via the photosystem II specific domain we are postulating could equilibrate with the inner aqueous space, thus accounting for the observations that the internal pH decreases as a function of increasing electron-transfer (protolytic reaction) rate (Gaensslen & McCarty, 1971; Rottenberg et al., 1972). The question of whether protons released by photosystem I interact with the CF_0 via another site-specific domain has not been dealt with here, and it remains to be investigated. Certainly the results of Ort et al. (1976) are consistent with some sort of direct communication between the coupling complex and protons released in both protolytic reactions, a communication pathway not obligatorily including the inner aqueous space. The data presented thus far pose intriguing questions about site specificity for proton interactions with the energy-coupling complex as well as questions about the mechanism of proton movement in the thylakoid membrane system.

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