Journal of Bioenergetics and Biomembranes, Vol. 16, No.1, 1984

Nonequilibration of Membrane-Associated Protons with the Internal Aqueous Space in Dark-Maintained Chloroplast Thylakoids

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Received May 1, 1983; revised June 27, 1983

Abstract

Isolated spinach thylakoids retain a slowly equilibrating pool of protons in the dark which are predominantly bound to buffering groups, probably amines, with low pKa values. We have measured the effects of permeant buffers, salts, sucrose, and uncouplers on the retention of the proton pool. Acetic anhydride, which reacts with neutral primary amine groups, was used to determine the protonation state of the amine buffering groups. It was previously shown by Baker *et al.* that the extent of inhibition of photosystem II water-oxidizing capacity by acetic anhydride and the increase in derivatization by the anhydride are proportional to, and dependent on, the deprotonated state of the amine buffering pool. Therefore, acetic anhydride inhibition of water oxidation activity may be used as a measure of the protonation state of the amine buffering pool. By this method it is inferred that protons, in a metastable state, were retained by membranes suspended in high pH buffer for several hours in the dark. When both the internal and external aqueous phases were equilibrated with pH 8.8 buffer, the proton pool was released only upon addition of a protonophore. The osmotic strength of the suspension buffer affected uncoupler-induced proton release while ionic strength had little influence. The acetic anhydride-sensitive buffering group(s) of the water-oxidizing apparatus had an apparent pKa of 7.8. We conclude that an array of protein buffering groups reside either within the membrane matrix, or in proteins at the membrane surface, not in equilibrium with the bulk aqueous phases, and is responsible for the retention of the proton pool in dark maintained chloroplasts.

Key Words: Proton processing; thylakoids; nonequilibrium; oxygen evolution; chemical modification.

Introduction

The pathway by which protons travel to and through the energy-coupling apparatus in chloroplasts remains an unresolved question. The Mitchell

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0145-479X/84/0200-0037$03.50/0

(1966) chemiosmotic hypothesis in its simplest formulation requires that the protolytic reactions of electron transport deposit protons directly into the bulk aqueous phase, leading to a transmembrane protonmotive force. Alternatively, Williams (1975) has proposed that proton movements leading to photophosphorylation are primarily restricted to the membrane itself. Experimental techniques, used with chloroplast systems, that can test these hypotheses include: (a) the effects of permeant buffers on the onset of ATP formation (Ort *et al.*, 1976; Graan *et al.*, 1981; Vinkler *et al.*, 1980; Davenport and McCarty, 1980); (b) kinetics of pH-indicating dyes that report the pH of the inner aqueous space (Junge *et al.*, 1978; Hong and Junge, 1983; Theg and Junge, 1983; Hope *et al.*, 1982); (c) measurement of the pH and electrical components of the protonmotive force compared to the free energy required to drive ATP synthesis (Giersch *et al.*, 1980; Hope *et al.*, 1982); (d) chemical modification of membrane-associated functional groups (Prochaska and Dilley, 1978; Baker *et al.*, 1981; and Tandy *et al.*, 1982). The reader will find that the works referred to in (a) and (b) above give quite different data patterns and hence interpretations. The evidence to date is quite divided, and it is clear that energy transduction mechanisms are not understood. Part of the conflicting data may have an explanation in membrane structural factors, especially in light of the recent work of Hong and Junge (1983). It appears from that work that the structural state of the thylakoids, as affected by freezing, for instance, can greatly influence the neutral red response, such that freshly prepared membranes show dye changes indicative of some type of localized proton processing. Frozen and thawed thylakoids, on the other hand, gave data consistent with proton release into the bulk inner aqueous phase.

Chemical modification probes can be useful tools to help elucidate membrane-protein interactions with protons (Dilley *et al.*, 1982). Acetic anhydride reacts rapidly with unprotonated amine groups but not with protonated amine groups. We have used this property to follow changes in the protonation state of amine functions of thylakoid membrane proteins under various conditions related to bioenergetic functions. Out of those experiments came the findings that thylakoid membranes have an array of 30–40 nmol · $(\text{mg chl})^{-1}$ of acetic anhydride-reactive groups (probably all or most of which are amine groups) with the following properties:

1. The "special pool" of buffering groups are behind the permeability barrier of the membrane. Chloroplasts freshly prepared in room light, but stringently dark-adapted subsequently, have the buffering group array in the protonated, anhydride-unreactive state. Either uncouplers, at low concentration, or a brief thermal treatment cause the loss of about 30–40 nmol H^+ · $(\text{mg chl})^{-1}$ with a concomitant increase in acetic anhydride-labeled groups and inhibition of water oxidation (Baker *et al.*, 1981, 1982). Homann and colleagues have also reported

on the properties of a metastable proton pool in thylakoid membranes (Theg and Homann, 1982, Theg *et al.*, 1982, Johnson *et al.*, 1983).

2. Some of the acetic anhydride-reactive groups described in (1) above are closely associated with the water-oxidizing apparatus (Baker *et al.*, 1981) and part of the array consists of the lysine 48 residue of the 8-kD CF_0 protein (Tandy *et al.*, 1981).
3. Either electron transfer-linked proton accumulation (Baker *et al.*, 1981) or ATPase proton pumping (Baker *et al.*, 1982) can convert the uncoupler or thermal treatment-induced state (deprotonated) to the protonated, anhydride-unreative state.

The association of some of the 30–40 nmol · (mg chl)$^{-1}$ of membrane buffering groups with the water-oxidizing apparatus and the 8-kD CF_0 protein suggests that these buffering groups may be involved in the bioenergetic functioning of thylakoid membranes. Obviously, the location of the amine group array with regard to the membrane structure is an important point to clarify. This report will deal with that question. The approach used involves varying both the outer and inner aqueous-phase pH and assaying the protonation state of the "special pool" of acetic anhydride-reactive groups by measuring the sensitivity of water oxidation to acetic anhydride inhibition.

Materials and Methods

Chloroplast Preparation

Chloroplasts were isolated from spinach following the method of Ort and Izawa (1973). The membranes, except where noted, were resuspended to give 2–3 mg chl/ml in a medium containing 5.0 mM HEPES–NaOH,[3] pH 7.5, 200 mM sucrose, 2 mM $MgCl_2$, and 0.5% defatted bovine serum albumin. The chlorophyll concentration was determined by the method of Arnon (1949).

Acetic Anhydride Modification

Chloroplasts were diluted to a concentration of 40 μg chl/ml in a lightshielded reaction vessel containing 50 mM HEPPS–NaOH, pH 8.6, 50 mM KCl, 2 mM $MgCl_2$, and 100 mM sucrose. When present, the nigericin concentration was 0.5 μM. The chloroplast suspension was stirred for 30 sec at 20°C prior to the addition of methanolic acetic anhydride to a final concentra-

[3]Abbreviations: AC_2O, acetic anhydride; chl, chlorophyll; DCMU, *N'*-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea; MV, methylviologen; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HEPPS, *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid; TAPS, tris[hydroxymethyl]methylaminopropanesulfonic acid; Tricine, *N*-[tris-hydroxymethyl]methyl glycine; FCCP, carbonyl cyanide [*p*-(trifluoromethoxy)phenyl]hydrazone.

tion of 3.5 mM. After 30 sec of treatment with acetic anhydride, the reaction was quenched by the addition of *N*-glycylglycine, pH 8.6, to a concentration of 50 mM. A 40–μg chl aliquot was then transferred to a cuvette containing 1 ml of the above anhydride-reaction buffer with a Clark-type oxygen electrode. Electron transport activities were measured as described in Baker *et al.* (1982). For control, unmodified chloroplasts, the quenching agent was added prior to the acetic anhydride. All reported electron transport values represent the maximal, uncoupled rate. Uncoupling was achieved by the addition of 2.0 μM nigericin after the quench reagent was added.

ΔpH Determination

The transmembrane ΔpH was determined by the amine distribution method using the equations of Rottenberg *et al.* (1972). The protocol followed was essentially that as described in O'Keefe and Dilley (1977). Chloroplasts were resuspended, following isolation, in the buffered solutions detailed in Tables I and II, allowed to equilibrate for 50 min on ice, then brought to room temperature. Either 10 μM [^{14}C]methylamine (25 mCi/mmol) or 10 μM [^{3}H]5-hydroxytryptamine (25 mCi/mmol) were added subsequently; 10 μM nigericin was added to the plus uncoupler samples. The suspension was incubated for an additional 10 min, at which time 0.1 ml (0.1 mg chl) was removed and centrifuged through a silicone oil layer consisting of Versilube F-50 and SF-96 in a ratio of 4:1. The chloroplast internal volume was determined with the silicone oil centrifugation technique using [^{3}H]H_2O with a correction for externally trapped aqueous phase using [^{14}C]sucrose (O'Keefe and Dilley, 1977).

Comments on the Use of Acetic Anhydride as a Probe for the Protonation State of Membrane Protein Amine Groups

A colleague proposed an alternative explanation for the uncoupler effects of increasing the anhydride labeling compared to a minus uncoupler control. The point made was that acetic anhydride reaction with a neutral amine in a localized domain would generate an acetic acid molecule (that is the correct chemistry). The acid could dissociate causing protonation of a neighboring NH_2 group, converting it to an unreactive amine, $-NH_3^+$, thus leading to the observed lower level of labeling in the absence of uncoupler. The effect of the uncoupler, in this view, would be to allow the escape of the proton associated with the acetic acid, thus keeping the second $-NH_2$ group in the neutral form. The assumption required for this point of view is that in the "native" state there is a pool of neutral $-NH_2$ groups trapped in a localized domain, rather than our interpretation that there is a pool of charged $-NH_3^+$ groups. This is

not a likely situation for the following reasons: (1) This argument requires that there be an array of sequestered amines in the neutral, $-NH_2$ form in dark, control membranes. However, we have directly measured (Baker *et al.*, 1981, 1982) the uncoupler release of about 20–30 nmol protons · (mg chl)$^{-1}$ from membranes, with a concomitant increase in the acetyl incorporation into membrane protein. That is consistent with the control state having protonated $-NH_3^+$ groups, and the uncoupler causing deprotonation. If the reason for the labeling increase upon uncoupler addition were as suggested above, there should be no correspondence between the uncoupler-released protons and subsequent increased derivatization. (2) Thermal treatment, in the absence of uncoupler, causes a similar increase in acetic anhydride labeling and inhibition of water oxidation, as that due to uncoupler (Baker *et al.*, 1981). Under those conditions, we showed that after the thermal treatment the addition of uncoupler no longer caused the efflux of protons, nor the labeling increase (Baker *et al.*, 1981). A brief illumination after the thermal treatment restored the membranes to a state in which uncoupler addition caused an efflux of protons (Baker *et al.*, 1982). Both the change in labeling of the membranes and the effects on water oxidation followed the pattern of increasing after the thermal transition and decreasing after brief illumination. (3) The environment of the differentially labeled $-NH_2$ groups seems quite hydrophobic, as indicated by the low pKa of the $-NH_2$ groups. Acetic acid released in such an environment may also have its pKa shifted by several pH units, but to the alkaline side. If so, it might be that the acid form could diffuse away as a neutral molecule, thus not being a source of protons for the suggested protonation of the $-NH_2$ group. (4) This alternative view does not take into account the observations that either light-dependent (Baker *et al.*, 1981) or ATPase-dependent proton pumping (Baker *et al.*, 1982) after a thermal (deprotonation) treatment returns the membranes to the state being less reactive with anhydride, and less inhibited, vis-a-vis water oxidation activity. It is most reasonable to conclude that the acetic anhydride effects do monitor the protonation state of amines.

Results

Effect of Incubation Time and Added Buffer pH on Anhydride Resistance

The question arises as to the location of the metastable "proton pool." If all 30–40 nmol H^+ · (mg chl)$^{-1}$ were located free in the inner aqueous thylakoid space, the pH would be around 2.5, assuming an internal volume of 10 μl · (mg chl)$^{-1}$. Clearly, this is not the case. Haraux and de Kouchkovsky (1979) showed that greater than 99% of all protons accumulated during light-driven proton uptake were bound to endogenous buffering groups in the

chloroplast. We may assume, similarly, that in the dark most of the protons which are released by uncoupler addition are derived from endogenous chloroplast buffering groups and/or internally accumulated buffer molecules.

If internally accumulated buffer were the source of these protons, then acetic anhydride resistance should be dependent upon the type of buffer, extent of buffer accumulation, and, therefore, incubation time of the chloroplasts in the buffer medium. Normally, chloroplasts are stored at pH 7.72 and 4°C. When an aliquot of these chloroplasts is diluted into the pH 8.6 reaction medium (at 20°C), a transient pH gradient is established. Complete decay of this gradient may take up to 120 sec ($t_{-1/2}$ = 10–20 sec), by analogy to the decay time for the electron transport-generated proton gradient. Since the anhydride treatment is usually performed within this time frame, it is important to know whether this transient ΔpH confers the anhydride resistance. Figure 1 compares the stability of anhydride resistance in chloroplasts suspended for various times up to 130 min at pH 7.72 and 8.64 at 4°C in media containing 5.0 mM HEPES (pKa = 7.84 at 4°C), and 5.0 mM HEPPS (pKa = 8.42 at 4°C), respectively.[4] Such stock suspensions were then diluted into 50 mM pH 8.6 buffer at 20°C and incubated for times from 15 sec to 10 min prior to addition of acetic anhydride. Dilution of the chloroplasts at pH 8.64 into pH 8.6 reaction medium (at 20°C) should not generate a significant proton gradient, and therefore little or no anhydride resistance should be observed if it is the transient ΔpH that confers the resistance. To further favor the equilibration of inner aqueous-phase protons, the time between the dilution into the pH 8.6 reaction medium and the addition of acetic anhydride was varied from 15 sec to 10 min. Resistance was retained as long as an uncoupler was not added, but was lost upon uncoupler addition. The effects of acetic anhydride did not depend on the storage pH of the chloroplasts nor on the buffer pKa. Thus, the anhydride resistance was *not* created by added buffers nor a transient pH gradient generated by the transition from one buffer medium to another.

Effect of Internal Chloroplast pH on Acetic Anhydride Inhibition

Although the internal and external-phase proton activities were at equilibrium under the conditions described in Fig. 1, there was a proton concentration gradient across the chloroplast membrane in the dark. Several groups (Rottenberg *et al.*, 1972; Graan *et al.*, 1981) have measured a transmembrane ΔpH in dark-maintained chloroplasts. Table I shows that chloroplasts suspended in buffer at pH 8.6 for 1 hr had an internal pH which was about 0.5 units lower than the external pH, as determined by the

[4]pKa values determined from the ΔpKa/°C values given in Good *et al.* (1966).

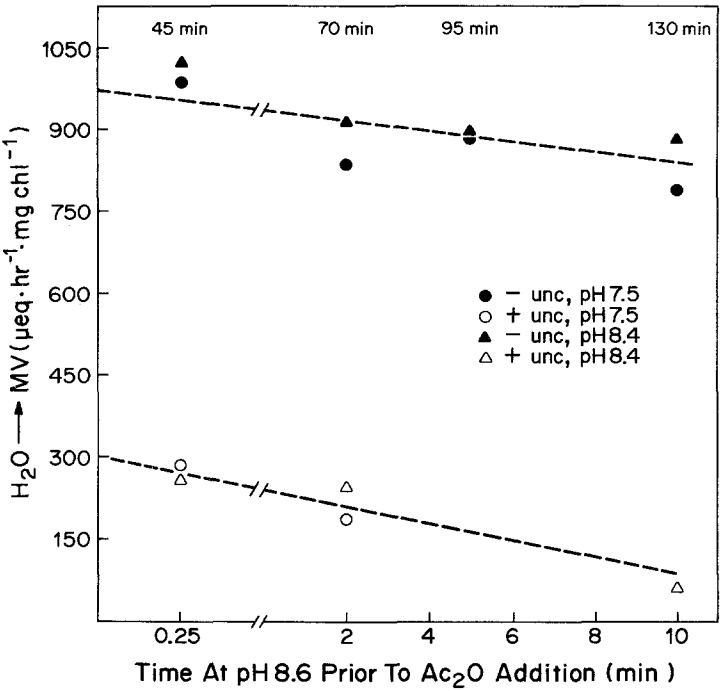

Fig. 1. Stability of acetic anhydride resistance in dark-held membranes. Two stock suspensions of chloroplasts were prepared by the methods of Ort and Izawa (1973): one using the usual resuspension medium of 5mM HEPES–NaOH, pH 7.5 (pH 7.72 at 4°C), 100 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, and 0.5% defatted bovine serum albumin, and the other having a similar composition, but using 5 mM HEPPS–NaOH, pH 8.4 (pH 8.64 at 4°C). Prior to treating these membranes with acetic anhydride, an aliquot of either stock was added to 2 ml of reaction medium at 20°C (giving 20 μg chl/ml) consisting of 50 mM HEPPS–NaOH, pH 8.6, 100 mM sucrose, 50 mM KCl, and 2 mM $MgCl_2$. The times in minutes at the top of the figure (45, 70, 95, or 130 min) indicate the times the stock membranes were present in their respective resuspension media. The abscissa indicates the additional time the membranes spent in the reaction medium, plus or minus 0.5 μM nigericin, before acetic anhydride treatment. Acetic anhydride modification and electron transport measurements were conducted as described under Materials and Methods.

distribution of either [^{3}H]5-hydroxytryptamine or [^{14}C]methylamine. Therefore, the "effective" pH at which anhydride resistance was measured may actually have been somewhat lower than the presumed pH 8.6 conditions. This dark ΔpH was stable even in the presence of 50 mM Tricine (Table 1, part b), which is known to equilibrate slowly into the lumen with a half-time of about 1.2 hr (Graan *et al.*, 1981). Table I also indicates that nigericin slightly decreased this ΔpH. It is unclear whether this apparent, dark ΔpH in the presence of high salt and uncoupler represents an actual transmembrane ΔpH

Table I. Determination of Dark ΔpH

Amine probe		Internal amine concentration (μM)	ΔpH	Anhydride resistant[c]
(a) Hydroxytryptamine[a]	No uncoupler	35	0.54	Yes
	Plus uncoupler	25	0.40	No
(b) Methylamine[b]	No uncoupler	37	0.57	Yes
	Plus uncoupler	21	0.32	No

[a] Chloroplasts were suspended at 1 mg chl/ml in a medium containing 10 mM HEPPS–NaOH, pH 8.6, 2 mM $MgCl_2$, 0.1 M sucrose, 0.5% bovine serum albumin, 10 μM [^{3}H]5-hydroxytryptamine, and, when present, 2.0 μM nigericin. Incubation conditions were as outlined in Materials and Methods. The internal aqueous volume was 10.0 μl/mg chl.

[b] Chloroplasts were suspended at 1 mg chl/ml in a medium containing 50 mM Tricine–NaOH, pH 8.6, 50 mM KCl, 50 mM sucrose, 2 mM $MgCl_2$, 10 μM [^{14}C]methylamine, and, when present, 10 μM nigericin. The internal aqueous volume was 5.1 μl/mg chl.

[c] Resistance of the chloroplasts to the inhibition of electron transport activity by acetic anhydride was determined as described under Materials and Methods.

or binding of the amine probe to the membrane. In either case, the mechanism which supports such an apparent ΔpH is unknown.

It is unlikely, however, that the 0.14 to 0.25 decrease in ΔpH caused by the uncoupler was responsible for the drastic difference in anhydride sensitivity. The results of an experiment which tests this more critically are described in Table II. In this case the chloroplasts were equilibrated with TAPS buffer at pH 8.8. Under these conditions the dark ΔpH was quite small and the effect of uncoupler on it was negligible. Yet, the anhydride-resistant state was sustained in the absence of uncoupler, even though the chloroplast internal pH was *higher* than the anhydride reaction medium pH. This result excludes the possibility that the uncoupler effect on the dark ΔpH was responsible for, or related to, the uncoupler effect on anhydride resistance. It further excludes

Table II. Dark ΔpH and Acetic Anhydride Resistance in Chloroplasts Equilibrated with TAPS[a] Buffer

	Electron transport activity ($\mu eq \cdot hr^{-1} \cdot mg\ chl^{-1}$)[b]	Internal pH	Dark ΔpH
No uncoupler	374	8.67	0.13
Plus uncoupler[c]	76	8.72	0.08

[a] Following isolation, chloroplasts were resuspended at 1 mg chl/ml in 15 mM TAPS–NaOH, pH 8.8, containing 2 mM $MgCl_2$, 50 mM KCl, and 10 μM [^{14}C]methylamine. The electron transport activities, following acetic anhydride modification, and dark ΔpH measurements were performed as described under Materials and Methods.

[b] The control, unmodified, electron transport rate was 530 $\mu eq \cdot hr^{-1} \cdot mg\ chl^{-1}$.

[c] For measurement of acetic anhydride inhibition of electron transport, 0.25 μM nigericin was added to the diluted chloroplasts just prior to anhydride treatment. For the measurement of dark ΔpH in the plus uncoupler case, 10 μM nigericin was added to the stock suspension of chloroplasts.

the possibility that anhydride resistance was conferred by a transmembrane pH gradient.

We conclude that neither protons derived from the inner aqueous phase, internally accumulated buffer, nor membrane buffering groups in equilibrium with the inner aqueous phase conferred the observed anhydride resistance. *Therefore, the source of the protons released by uncoupler addition, resulting in the anhydride effects on water oxidation, must be an array of buffering groups not in equilibrium with the bulk aqueous phase, probably contained within sequestered regions of membrane proteins.*

Effect of Osmolarity and Ionic Strength on Acetic Anhydride Inhibition

The capacity of uncouplers to release protons from the membranes is apparently dependent upon the osmolarity (but not ionic strength) of the reaction medium. Figure 2 demonstrates that the ability of 0.5 μM nigericin to sensitize membranes in the dark to acetic anhydride inhibition was diminished by high concentrations of sucrose. In 0.6 M sucrose there was only a 30% difference in the extent of inhibition by acetic anhydride (*plus* versus *minus*

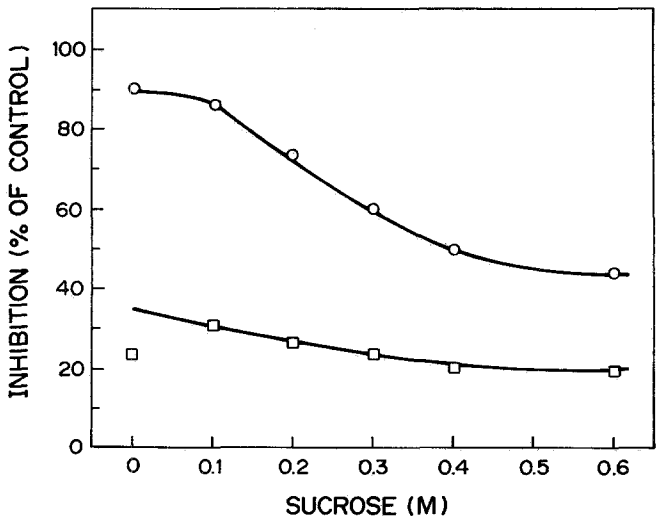

Fig. 2. Effect of osmolarity on extent of acetic anhydride inhibition of electron transport. Chloroplasts were treated with acetic anhydride in reaction media containing the indicated concentration of sucrose. Acetic anhydride modification was performed as described under Materials and Methods, except the chloroplasts were incubated 1 min in the reaction media, instead of 30 sec, in the presence (○) or absence (□) of 0.5 μM nigericin, before the addition of acetic anhydride. The control, unmodified, electron transport rate of 1360 μeq · (hr · mg chl)$^{-1}$ was unaffected by sucrose concentration.

Table III. Effect of KCl and Valinomycin on the Inhibition of Electron Transport Activity by Acetic Anhydride[a]

Treatment conditions	Electron transport activity (μeq · hr^{-1} · mg chl^{-1}
(A) Control	940
No uncoupler	660 (30)[b]
Plus VAL.	660 (30)
Plus NIG.	120 (87)
(B) High KCl, control	700
High KCl, no uncoupler	350 (50)
High KCl, plus VAL.	310 (56)
High KCl, plus NIG.	100 (86)

[a]Conditions were as described in Materials and Methods. The buffer consisted of 50 mM HEPPS–NaOH, pH 8.6, 50 mM KCl, 2 mM $MgCl_2$, 100 mM sucrose and, when present, either 1.0 μM nigericin (NIG.) or 0.5 μM valinomycin (VAL.) (added prior to the acetic anhydride treatment). For the control samples, *N*-glyclyglycine was added prior to the acetic anhydride. The potassium chloride concentration during the "high KCl" treatment was 0.425 M instead of 50 mM.

[b]The values in parentheses represent the percentage inhibition of electron transport.

uncoupler), which is small compared to the 80% difference seen in 0.1 M sucrose. High osmolarity similarly decreased the ability of a thermal transition to create the anhydride-sensitive state (data not shown). Theg *et al.* (1982) and Johnson *et al.* (1983) also observed that high osmolarity decreased the extent of gramicidin-induced proton release from dark-maintained thylakoids.

Contrary to the effect of high osmotic strength on the release of protons from the membrane, high ionic strength, in spite of the accompanying osmotic potential, did not prevent the release of protons by uncoupler. This is concluded from the observation that high KCl concentrations (0.425 M) did not prevent anhydride inhibition of electron transport (Table III, part B). In fact, the high salt conditions may have slightly increased the sensitivity of the membranes to anhydride. In the absence of uncoupler, high KCl concentrations increase the amount of inhibition to 50%, compared to the 30% inhibition under (relatively) low salt conditions.

Valinomycin did not increase the anhydride sensitivity of the membranes in the presence of 0.05 or 0.425 M KCl (Table III). This indicates that the membrane-bound protons were not influenced by a transient membrane potential of several hundred millivolts induced by the K^+ influx which occurred upon addition of valinomycin to the high-KCl suspension medium.

pH Dependence of the Acetic Anhydride-Sensitive State

Figure 3 shows the anhydride inhibition of water oxidation activity as a function of the buffer pH in the presence or absence of uncoupler. The

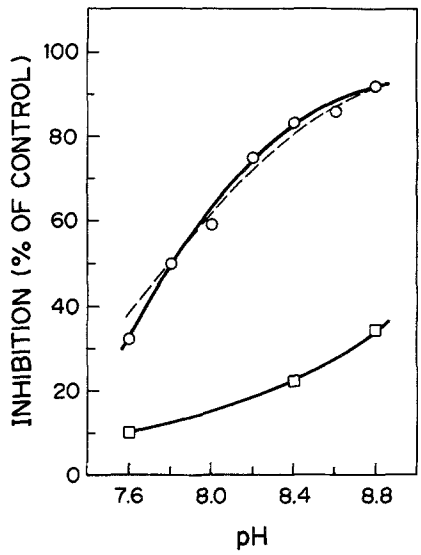

Fig. 3. Extent of acetic anhydride inhibition of electron transport activity as a function of pH. Chloroplasts were treated with acetic anhydride in reaction media containing 0.1 M sucrose, 50 mM KCl, 2 mM $MgCl_2$, and 50 mM HEPPS–NaOH titrated to the indicated pH, either in the presence (O) or absence (□) of 0.5 μM nigericin, as described under Materials and Methods. The control, unmodified, electron transport rate ($H_2O \rightarrow MV$) was 1020 μeq · (hr · mg chl)$^{-1}$.

inhibition in the absence of uncoupler required a much higher pH (i.e., 50% inhibition at pH $\geq$ 9.0) than when uncoupler was present. The pH curve for inhibition in the presence of uncoupler resembled the titration curve of a dissociating group with pKa of about 7.8. A theoretical curve (dashed line of Fig. 3), calculated from the Henderson–Hasselbach equation (assuming pKa = 7.8), is superimposable with the experimental data. This suggests that anhydride inhibition of water oxidation activity resulted from modification of an amine, or group of amines, with a single apparent pKa that is more acidic than the general population of anhydride-reactive groups. The latter point is supported by the previous observations that the number of protons released from chloroplast thylakoid membranes, as detected by acetic anhydride labeling (Prochaska and Dilley, 1978) or directly with a pH electrode (Theg *et al.*, 1982), increased more sharply with pH than the (top) curve shown in Fig. 3. The labeling of the membrane increased with pH, with a steep nonlinear increase beginning at about pH 8.2–8.3. Therefore, it seems likely that the sensitive functional group(s) involved in water-oxidation inhibition are a relatively small part of the total anhydride-reactive groups.

Discussion

The results presented in this paper demonstrate that chloroplast membranes retain a pool of protons in the dark, probably bound to amine buffering groups, which are not in rapid equilibrium with either the internal or external

aqueous phases. Figure 1 clearly indicates that protons are held by the thylakoids in a metastable state for more than 2 hr in 5 mM, high-pH buffer, a time period equal to about two half-times of equilibration of buffer molecules between the inner and outer aqueous phases (Graan *et al.*, 1981). The equilibration rate of bulk aqueous-phase protons is much faster, of course, having a half-time less than 1 min. The data in Table II, from an experiment using 25 mM TAPS buffer at pH 8.8, more critically tests our contention that the membrane-associated protons are not in equilibrium with the aqueous phases. In that experiment, assuming the amine distribution technique represents an adequate measure of the transmembrane ΔpH, the internal and external aqueous pH values were essentially identical and independent of added uncoupler. Yet, the membranes retained the acetic anhydride-resistant state (a measure of the metastable, bound-proton pool) until uncoupler was added.

The effects of high sucrose concentration in decreasing anhydride sensitivity (Fig. 2), but the opposite effect of high KCl concentration (also an osmoticum) (Table III), indicate that factors more complicated than simple osmotic effects are responsible for the observed changes in acetic anhydride inhibition. Chloroplast thylakoids respond as osmotically competent organelles in either sucrose or KCl (Dilley and Rothstein, 1967). Decreasing the water potential with high sucrose concentrations should have the effect of increasing the salt concentrations within any osmotically active subcompartments, an effect seemingly in the same direction as would follow from increasing the KCl concentration in the suspension; yet the effects of the two treatments are quite different. This is consistent with the anhydride-sensitive groups not being localized in the bulk, inner aqueous space. These effects may be due to alterations in membrane protein–protein or protein–lipid interactions, which are more sensitive to external than internal ionic strength effects. A minimal conclusion can be that the effects are consistent with the working hypothesis that amine groups function as reversible buffering groups within sequestered domains. This is in agreement with independent results of Johnson *et al.* (1983).

The buffering groups, probably amines, which constitute the membrane-bound proton pool apparently represent a heterogeneous population. This is suggested by the wide pH range in which protons are released from the membrane (cf. Fig. 3, Theg *et al.*, 1982, and Fig. 1, Prochaska and Dilley, 1978). The acetic anhydride inhibition of electron transport implies that there is a subpopulation of these amines, having a unique pKa of about 7.8, that must be associated with the oxygen-evolving apparatus of photosystem II (Fig. 3). Theg and Junge (1983) found a similar pKa for a neutral red absorbance change which was interpreted as due to Photosystem II-associated buffering groups in the membrane. It is also of interest to note that the pH

dependence of Cl^- release (uncoupler dependent) from thylakoids resembles that of the anhydride inhibition (Theg and Homann, 1982).

The low apparent pKa emphasizes the unusual environment of these amines. Either a hydrophobic environment or an electrostatic influence of nearby positive charges can cause the pKa of lysine ϵ-amino groups to drop from the normal value of about 10.7 to as low as 6 (Kokesh and Westheimer, 1971). Either influence may play a role in the stabilization of the membrane-associated proton pool. Obviously, the membrane matrix itself provides a hydrophobic environment. Additionally, however, it is of interest that three membrane proteins closely associated with the photosystem II water-oxidizing apparatus (M_4 33,000, 24,000 and 18,000) are rich in lysine residues (Yamamoto *et al.,* 1983). Those same three proteins show very large light-dark acetic anhydride labeling changes (Laszlo *et al.,* in preparation), suggesting that those lysines are part of the array of buffering groups within the membrane. It is possible that protons released in water oxidation are initially deposited into a sequestered domain containing the anhydride-sensitive lysine groups. The latter point would be consistent with recent findings of Theg and Junge (1983) which indicate that uncoupler-induced proton depletion of membranes results in a seven- or eight-flash lag in the development of fast neutral red dye changes.

Kell (1979) has proposed that the Stern–Grahame water layer adjacent to the membrane surface may present a diffusion barrier to protons, keeping them near the surface. Permeant ions such as valinomycin *plus* K^+ disrupt this layer and permit rapid equilibration of protons with the bulk aqueous phase. Such a mechanism cannot, however, be invoked to explain the lack of equlibration of protons with dark-maintained chloroplast membranes because the presence of valinomycin *plus* K^+ did not produce the anhydride-sensitive state (Table III).

The data presented above seem to us strong evidence for the existence of a sequestered, or buried, array of proton buffering groups within the thylakoid membrane. Membrane-protein amine groups are the most likely functional groups involved. There are no known amino lipids in thylakoid membranes (Nichols and James, 1968). We have identified the lysine 48 amine group of the 8-kD CF_0 protein as one of the target groups constituting up to about 1–2 nmol · $(mg\ chl)^{-1}$ of acetyl labeling out of the 30 nmol · $(mg\ chl)^{-1}$ labeling that is modulated by uncoupler addition and light or dark conditions (Tandy *et al.,* 1982). The location and/or arrangement of the other buffering groups of the sequestered proton pool remains to be elucidated. It is also unclear how uncouplers of quite different structure, for example, FCCP, gramicidin, nigericin (Baker *et al.,* 1981), or a thermal treatment interact with the membrane to release the metastable protons.

Previous work using either diazonium benzene sulfonate (Giaquinta *et*

al., 1975) or acetic anhydride and iodoacetate (Prochaska and Dilley, 1978; Baker *et al.*, 1981, 1982) had led to the suggestion of localized, perhaps intramembrane, proton interactions with thylakoid membrane proteins. That work was based on differences between light and dark conditions and drew heavily on the interpretation based on differences between effects of photosystem I and photosystem II partial reactions in potentiating the labeling patterns and inhibition of electron transport. More recent work, using acetic anhydride, has revealed that under some conditions the labeling and inhibition effects potentiated by the photosystem I or II partial electron transport systems can be similar (Baker, 1983). That result could be interpreted as indicating that the proton effects elicited by the two separate photosystems were originating in the inner aqueous space, rather than the interpretation presented here which favors a localized proton interaction. The present results, employing only dark incubation conditions, gives unequivocal support to the original interpretation, namely that the chemical-modification results with acetic anhydride are due to localized proton–membrane protein interactions in a sequestered domain.

The possible involvement of the sequestered proton domains in such bioenergetic functions as proton movement in the membrane into the ATP-forming complex is a pertinent question, presently under study. Obviously, with a pKa near 7.8, the localized proton binding does not reflect interactions with protons necessarily having a large electrochemical potential gradient. The point of view we presently favor follows the ideas of Nagle and Morowitz (1978), in that proton-binding groups may be involved in a mechanism for allowing localized proton movement within the thylakoid membrane. The involvement of the 8-kD CF_0 protein (Tandy *et al.*, 1982) as a part of the localized proton domain suggests the possibility that the domain may involve proton movement into the ATP-generating system.

Acknowledgments

The authors thank Ms. Lucy Winchester for help in preparation of the manuscript. This work was supported in part by NSF grant PCM 76-0164 and by NIH grant GM 19595.

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