Journal of Bioenergetics and Biomembranes, Vol. 21, No. 4, 1989

Chloroplast Thylakoid Proteins Associated with Sequestered Proton-Buffering Domains. Plastocyanin Contributes Buffering Groups to Localized Proton Domains

F. C. T. Allnutt,[1] E. Atta-Asafo-Adjei,[1] and R. A. Dilley[1]

Received April 10, 1989; revised June 5, 1989

Abstract

Thylakoid membrane proteins are organized so as to shield 30–50 nmol H^+ (mg Chl)$^{-1}$ from freely equilibrating with either the external or the lumen aqueous phases. Amine groups provide binding sites for this metastable buffering array and can be quantitatively measured by acetylation using [^{3}H]acetic anhydride. The principle of the assay is that a metastable acidic domain will have relatively less of the reactive neutral form of the amine compared to the amount present after addition of an uncoupler. The extent of the acetylation reaction is strongly influenced by whether the lumen pH comes to complete equilibrium with the external pH prior to adding the acetic anhydride. Determination of the lumen pH by [^{14}C]methylamine distribution after the standard 3 or 5 min equilibration in pH 8.6 buffer indicated that the lumen may have been 0.2 to 0.3 pH more acidic than the external phase. This effect was taken into account by determining the pH dependence, in the pH 8.2–8.6 range, of acetylation of the membrane proteins studied, and the labeling data were conservatively corrected for this possible contribution. Experiments were carried out to identify the thylakoid proteins that contribute such metastable domain amine groups, using the above conservative correction. Surprisingly, plastocyanin contributes buried amine groups, but cytochrome *f* did not give evidence for such a contribution, if the conservative correction in the labeling was applied. If the correction was too conservative, cytochrome *f* may contribute amines to the sequestered domains. The new methodology verified earlier results suggesting that three Tris-releasable photosystem II-associated proteins also contribute significantly to the sequestered amine-buffering array.

Key Words: Localized protons; thylakoid proteins; metastable sequestered buffering groups.

[1] Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907.

0145-479X/89/0800-0535$06.00/0

Introduction

Chloroplast thylakoid membranes, de-energized and in the dark, retain protons within metastable sequestered domains. The addition of uncouplers to de-energized thylakoids suspended at pH 8.6 results in the release of 30–50 nmol $H^+ \cdot (mg\ Chl)^{-1}$ from this sequestered buffer array with an apparent pK_a near 7.5 (Baker *et al.*, 1981, 1982; Theg *et al.*, 1982; Laszlo *et al.*, 1984a; Pfister and Homann, 1986; Dilley *et al.*, 1987). The source of these protons must be a membrane phase domain that is not in equilibrium with either the aqueous lumen or the external phase (Dilley *et al.*, 1987). These domains can be depleted of protons, in the dark, by addition of low concentrations of uncouplers such as nigericin, CCCP, or desaspidin, or by mild heat (Baker *et al.*, 1981, 1982). Following proton depletion with CCCP[2] or desaspidin, addition of BSA binds the uncoupler and removes it from the membrane, after which the domains can be efficiently reprotonated by H^+ pumping *via* the redox reactions (Dilley and Schreiber, 1984; Theg *et al.*, 1988) or through ATPase H^+ pumping (Baker *et al.*, 1982). The above properties take on heightened interest in view of other data demonstrating a correlation between the protonation state of the domains and the number of single turnover flashes required to energize thylakoids for ATP formation (Dilley and Schreiber, 1984; Theg *et al.*, 1988) [i.e., the domain protons were shown to be the first protons driven into the CF_0–CF_1 complex by the electric field component of the protonmotive force (Theg *et al.*, 1988)]. In the absence of an electric field and with an external pH near 8, the protons associated with the pK_a 7.5–7.8 amine array are not energetically competent to initiate ATP formation, because a ΔpH of near 2.3 units is required (Beard and Dilley, 1988). The amine groups could, however, provide charge relay sites for proton movement within the putative localized pathway. A much larger array ($\approx$ 200 nmol $(mg\ chl)^{-1}$ of lower pK_a carboxyl groups is also contained in the sequestered domains, which may contribute energetically competent protons for ΔpH-driven ATP formation (Beard *et al.*, 1988; Beard and Dilley, 1988). Less is known about the carboxyl group buffering array, but it appears to not hold protons as tightly as the higher pK_a amine buffer array (Beard and Dilley, 1988). The above data suggest a possible role of the sequestered proton buffering domains in thylakoid bioenergetics (for a review, cf. Dilley *et al.*, 1987), and give renewed impetus for the study of membrane components which may be associated with the sequestered domains.

Laszlo *et al.* (1984b) indicated that about nine thylakoid proteins contribute amine proton-buffering groups to the sequestered domains.

[2] Abbreviations: cyt *f*, cytochrome *f*; CCCP, carbonylcyanide-*m*-chlorophenyl hydrazone; OEC, oxygen-evolving complex; PC, plastocyanin; Nig, nigericin.

Components so far identified include the 8 kDa CF_0 subunit (Tandy *et al.*, 1982), the three extrinsic membrane proteins (18, 22, and 33 kDa subunits) associated with the oxygen-evolving system, and the light-harvesting complex (LHC) (Laszlo *et al.*, 1984b). In this report we confirm those studies using more refined methods, as well as test other proteins to determine their role in the proton domains. Vis-a-vis proton diffusion from sites of proton release in the redox chain to the CF_0–CF_1 complex via sequestered domains, it seems particularly pertinent to study proteins closely associated with proton production and utilization. Hence, we here concentrate on the oxygen evolving system proteins and those of the plastoquinol-oxidizing cytochrome b_6/f complex. Plastocyanin was studied as a comparison, it was believed, to a protein thought to be freely diffusible in the lumen. The CF_1 polypeptides provided another type of control, being largely on the external surface of the membrane.

Materials and Methods

Chloroplasts were isolated from market spinach as described by Ort and Izawa (1973), and stored in 200 mM sorbitol and 3 mM $MgCl_2$, with either 5 mM MES-KOH (pH 6.5) or 5 mM HEPES-KOH (pH 7.5) as indicated. Chlorophyll and protein content were assayed as described previously (Laszlo *et al.*, 1984b).

The assay for proteins associated with sequestered domains is based on measuring the increase in [^{3}H]acetic anhydride derivation that occurs after the thylakoids lose the sequestered protons following addition of the uncoupler nigericin (Baker *et al.*, 1981; Laszlo *et al.*, 1984b). The nigericin-induced proton efflux shifts the amine dissociation reaction, $pK_a \approx 7.7$, to the right ($-NH_3^+ = -NH_2 + H^+$), leading to a greater anhydride reactivity (only the $-NH_2$ form reacts with acetic anhydride). The assay is the difference in [^{3}H]acetyl incorporation between a thylakoid sample treated when the domains are more fully protonated (dark conditions, pH 8.6, no nigericin), compared to a sample given nigericin (to dissipate the domain protons) before the acetylation step. This assay depends on there being an acidic localized domain (before addition of uncoupler) but having the lumen phase equilibrated with the external phase. Under this condition, the uncoupler-induced increase in anhydride reactivity should indicate only localized domain amine groups. A possible concern would be a contribution to labeling if the lumen phase had not reached equilibrium with the external medium. In that case, on addition of uncoupler a residual ΔpH may be dissipated and result in artifactual differences in labeling by [^{3}H]acetic anhydride. Because the lumen seems to remain slightly more acidic (about 0.2 pH units) than the

pH 8.5 external phase in the absence of uncoupler, even with 5 min equilibration time, this possible contribution to the differential labeling has to be assessed. One way to correct for the residual pH contribution is to measure the pH dependence of the anhydride labeling of each protein we work with, and relate that to the estimated lumen pH difference for the control and plus uncoupler labeling treatments. If the observed differential labeling *in situ* is significantly larger for a given protein than that expected for the dark, residual Δ pH (calculated from isolated protein labeling data), we then assign the additional labeling to amine groups sequestered in the domains.

Acetic anhydride acetylation of thylakoid membranes was performed at 20°C utilizing 0.2 mM acetic anhydride as previously described (Laszlo *et al.*, 1984a). Using 0.2 mM acetic anhydride for 30 sec permits trace levels of labeling that is proportional to the complete acetylation of reactive groups (Laszlo *et al.*, 1984b). The lower concentration of acetic anhydride was used because higher acetylation levels led to changes in mobility of some proteins in the gel electrophoresis step. Thylakoids were suspended at 50–100 μg Chl ml^{-1} in reaction medium consisting of 100 mM sucrose, 50 mM EPPS-KOH, pH 8.6, 3 mM $MgCl_2$, and 50 mM KCl. These experiments were performed in a darkened room with the chloroplast suspension shielded from stray light. The chloroplasts were equilibrated 3 or 5 min prior to addition of [^{3}H]acetic anhydride. After exactly 30 sec of acetylation the reaction was quenched by addition of 50 mM N-glycyl glycine (pH 8.6), followed by centrifugation at 8000 *g* for 15 min. After resuspending and washing twice in fresh medium, the pellets were used for protein isolations as described below.

pH dependence of isolated protein acetylation. Isolated PC, partially purified cyt b_6/f complex, and the isolated OEC polypeptide were derivatized with [^{3}H]acetic anhydride over a pH range from 7.0 to 9.0 to estimate the possible contribution of the slightly acidic lumen to the differential anhydride labeling. Plastocyanin was acetylated in a medium of 50 mM TAPS, 50 mM MOPS, 50 mM KCl, and 2 mM $MgCl_2$, with the pH adjusted with NaOH. Isolated cyt b_6/f complex was acetylated in a number of different media to determine if the various resolubilization methods exposed additional reactive amines. Since all methods tried gave similar results, cholic acid was used to solubilize the complex for these measurements as described in the figure legends. Acetylation was with 0.2 mM [^{3}H]acetic anhydride for 30 sec at 20°C; the reaction was quenched with 50 mM N-glycyl glycine, pH 8.5, and the proteins were precipitated with 95% acetone and washed again with 95% acetone; the incorporated radioactivity was determined by liquid scintillation counting.

The pH dependence of acetic anhydride modification of the isolated OEC polypeptides (cf. below for the isolation procedure) was performed as described above except that the nig and valinomycin were left out of the

reaction medium (residual detergent from the isolation steps would dissipate any proton gradients). After labeling with [^{3}H]acetic anhydride the OEC polypeptides were pelleted with 10% TCA, washed with 95% acetone, and dissolved in SDS solubilizing buffer for electrophoresis.

Isolation of the cyt b_6/f complex was described by Black *et al.* (1987). The starting material was about 100 mg Chl as class II thylakoids, which were either acetylated with [^{3}H]acetic anhydride or unlabeled anhydride depending on the experiment. Because of the small amount of starting material, the final purification step used by Black *et al.* (1987) was not attempted; instead the preparation was terminated after the first calcium phosphate column chromatography step. Final separation of the polypeptides relied on SDS-PAGE.

Isolation of PC was as described by Ellefson *et al.* (1980) except that the starting material was 100 mg Chl as Class II chloroplasts as described above. The preparation was stopped after the DEAE-cellulose column, and final separation step was by gel electrophoresis. For the isolation of the purified PC used in control experiments the entire Ellefson *et al.* procedure was followed.

The isolation of the CF_1 was according to Kamienietzky and Nelson (1975).

Isolation of the extrinsic OEC polypeptides was from either [^{3}H]acetic anhydride labeled or unlabeled thylakoids by the method of Berthold *et al.* (1981). Thylakoids were isolated and stored in the pH 6.5 MES buffer as decribed above. Acetylation with 0.2 mM [^{3}H] acetic anhydride was carried out at pH 8.5 either with or without prior addition of 0.5 μM nig and 0.5 μM valinomycin in reaction conditions as described above for the acetylation step. Acetylation as a function of pH between 8.0 and 8.6 was carried out in a similar way except that 0.5 μM nig and 0.5 μM valinomycin were present in all the samples 30 sec prior to addition of the [^{3}H]acetic anhydride.

A Tris wash at pH 9.0 (Laszlo *et al.*, 1984b) was employed to release the OEC polypeptides. Similar results were obtained using the $CaCl_2$ method (Ono and Inoue, 1983). Extracted polypeptides were dialyzed against 5 mM Tricine, pH 9.0 (for the Tris wash), or 5 mM Mes, pH 6.5 (for the $CaCl_2$ wash), then concentrated by freeze drying. In the case of acetic anhydride labeling of thylakoids prior to isolating the extrinsic OEC polypeptides, the lyophilized proteins were suspended in H_2O and precipitated with 10% TCA, and the pellet washed in 95% acetone, pelleted again, and taken up in 10% SDS prior to loading on the gels for the SDS-PAGE preparation. The gel bands corresponding to the extrinsic OEC polypeptides were excised and assayed for radioactivity as described below.

Protein electrophoresis by the SDS-PAGE method was as described by Laemmli (1970), either with or without 6 M urea. For electrophoresis of PC, the method of Hoober (1970) was applied. Gels from the cyt b_6/f preparations

were stained for heme-containing proteins on non-urea gels (Thomas *et al.*, 1976). Coomassie Blue-stained gels were scanned for band intensity using an Isco model 1312 gel scanner. Bands were excised with a razor, digested, and decolorized with H_2O_2 at 80°C, and the radioativity was measured by scintillation counting. A BioLab planimeter was used to determine the area under peaks of the density scan of Coomassie Blue-stained gels. The acetylation level of a given protein was expressed as radioactivity (cpm) per area of the stained gel band. This was considered a more accurate criterion than relating the total counts recovered in a protein band to the amount of protein loaded on the gel, owing to there occasionally being incomplete entry of the loaded protein into the gel.

Determination of the thylakoid transmembrane ΔpH was by the distribution of [^{14}C]methyl amine as previously described (Rottenberg *et al.*, 1972). The medium used for this determination was the same as that used for acetylation of thylakoids, as previously described.

Results

Establishing Conditions for the Acetic Anhydride Labeling Assay

The assay for detecting amine groups associated with the sequestered domain requires that the thylakoid lumen pH be close to that of the alkaline (pH 8.6) suspending phase prior to addition of the [^{3}H]acetic anhydride. In this resting state the sequestered domain remains metastably more acidic than the two bulk phases for tens of minutes, albeit slowly losing protons to the more alkaline phases (Laszlo *et al.*, 1984a). Therefore, in the metastable condition, the amine groups in the domains ($pK_a \approx 7.5$) are relatively protonated and less reactive with acetic anhydride than would be the case at pH 8.6. Addition of an uncoupler in the dark prior to acetylation induces rapid proton loss from the domains (Baker *et al.*, 1982) and conversion of the amines to the neutral, reactive form, thereby increasing the labeling level.

We followed the suggestion of Pfister and Homann (1986), who showed that using pH 6.5 Mes buffer rather than the commonly used higher pK_a buffers (Tricine or HEPES) in the thylakoid stock suspension medium minimizes the contribution of protons, by storage buffers present in the lumen, to uncoupler-dependent proton efflux. In addition, we measured the lumen pH using the distribution of [^{14}C]methylamine after either a 3 or 5 min incubation at pH 8.6. For comparison we also measured the lumen pH of similarly suspended thylakoids that had been stored at pH 7.5 in buffer with 5 mM Hepes as the buffering agent. The conditions used for the ΔpH assay were identical to those used for the incubation period of an [^{3}H]acetic

Table I. Δ pH Determined by [^{14}C]Methylamine Distribution of Dark-Equilibrated Thylakoids in the Presence or Absence of 0.5 μM Nigericin[a]

Storage buffer	Incubation time (min)	Internal pH		Δ pH induced by nig
		− nig	+ nig	
Hepes, pH 7.5	3	7.79	8.01	0.22
Hepes, pH 7.5	3	7.50	7.77	0.27
Mes, pH 6.5	3	7.60	7.86	0.26
Mes, pH 6.5	5	7.48	7.67	0.19

[a]Thylakoids were prepared as described in the Materials and Methods Section and suspended for storage in either 5 mM Hepes, pH 7.5, containing buffer or buffer having 5 mM Mes, pH 6.5, as indicated. After diluting the thylakoids into the reaction medium for determining [^{14}C]methylamine distribution across the membrane (see Materials and Methods for the reaction medium composition and other details), the thylakoids were incubated 3 or 5 min in the dark at pH 8.6 prior to centrifuging. The largest sample standard deviation was 0.07 pH units. All paired values were different at the 98% confidence level.

anhydride labeling experiment. Table I shows that at 20°C the lumen was slightly more acidic than the suspending phase, but only part of that apparent acid gradient was dissipated by nig. After a 3 or 5 min incubation at pH 8.6 the acidity dissipated by nig is equivalent to a Δ pH of 0.2 to 0.3 units. We found no significant difference in the lumen pH equilibration between 3 and 5 min of incubation. Given that the $t_{1/2}$ for H^+ gradient relaxation after the light-dependent H^+ uptake is about 10 sec at 20°C, pH equilibration should be largely completed after six or seven half-lives, which is less than the 3 min period given in the shortest incubation time used here. The Δ pH remaining after nigericin addition was 0.6 to 0.9 pH units, but some of that probably represents binding of methylamine to the membranes and likely is not a true Δ pH. Graan *et al.* (1981) used similar techniques for thylakoid Δ pH determination but with a 15 min incubation at 4°C, and found the lumen pH unchanged with or without uncoupler added. They also reported a 0.3–0.4 unit Δ pH (acid inside) that was resistant to uncoupler.

While the Δ pH which remains after nig addition probably is not a true transmembrane Δ pH, there is about 0.2 to 0.3 Δ pH which is dissipated by addition of uncoupler and directly impacts our assay. The nig-dissipated Δ pH represents a possible acidic condition that may cause lumenal-facing amine groups to be more protonated than if the groups were exposed to the external phase. Thus, there may be some contribution to the nig-induced protein acetylation owing to lumen-facing amine groups in addition to the sequestered domain groups. To assess this contribution, the pH dependence of the acetylation of PC, cyt *f*, and the OEC extrinsic polypeptides was determined. Those pH-dependent acetylation changes estimated as caused by a possible 0.3 Δ pH gradient pressure in the minus nig case can be compared

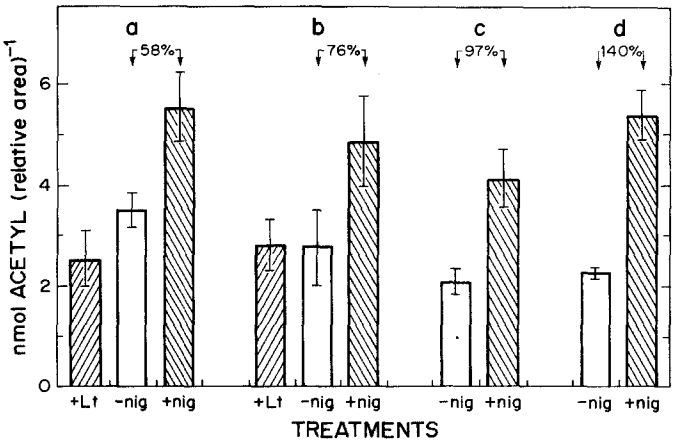

Fig. 1. [^{3}H]acetyl incorporation into PC, which was isolated from spinach thylakoids previously acetylated with 0.2 mM [^{3}H]acetic anhydride under complete darkness with (+ nig) or without (− nig) 0.5 μM nig or under saturating light with 0.5 μM nig (+ Lt). Experiments a, b, and d were done with thylakoids resuspended in Hepes, pH 7.5, resuspension medium and equilibrated for 3 min prior to modification. Experiment c was done with Mes, pH 6.5, resuspension medium and equilibrated for 5 min prior to acetylation. All other conditions were as described in the Materials and Methods section. Data represent the mean of at least three replicates which were normalized by the density of the Coomassie blue-stained PAGE band. The crossbars represent the sample standard deviation.

to the acetylation changes which occurred in the respective proteins following nig addition to thylakoids. Nig-induced acetylation greater than that accounted for by 0.3 unit Δ pH can thus be taken as a conservative estimate of amine groups that contribute to the sequestered domain buffering.

Plastocyanin Derivatization with [^{3}H]Acetic Anhydride

Plastocyanin, isolated from acetic anhydride-labeled thylakoids, showed a nig-induced labeling increase of 58 to 140% (Fig. 1). When Mes pH 6.5 thylakoid storage medium (Fig. 1d) was substituted for the pH 7.5 Hepes storage medium (Fig. 1a, b, c), the extent of labeling was similar and the same labeling trend was observed. When isolated PC was modified with acetic anhydride, no significant increase in labeling was observed on addition of nig (data not shown).

The pH dependence of purified PC acetylation (Fig. 2) indicates that if the lumen pH were 0.3 units more acidic than the external pH of 8.6, one could expect a 16% increase in acetylation upon addition of nig. Clearly this is much less than the measured 58–140% labeling difference in PC. For a nig-induced 100% labeling increase in PC to be explained as owing to the residual lumen pH before nig addition, there would have to be about a 1 pH

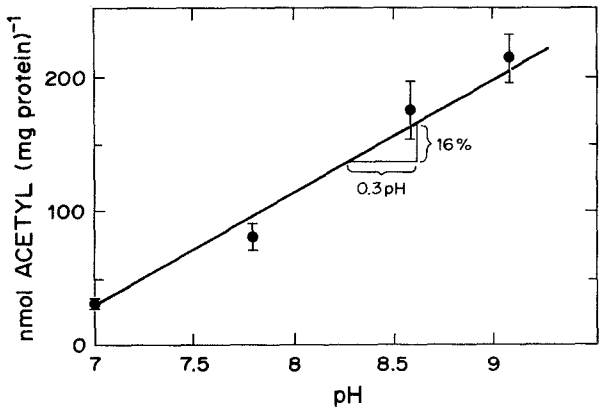

Fig. 2. The incorporation of [^{3}H]acetyl into purified PC as a function of the pH of the reaction buffer. Purified PC (90 μM) was acetylated with 0.2 mM [^{3}H]acetic anhydride in a medium containing 50 mM each of sucrose, TAPS, MOPS, and KCl and 2 mM $MgCl_2$. All other conditions were as described in the Materials and Methods section. Data represent the average of at least three replicates normalized by protein content.

unit acidic condition maintained in the lumen during the dark incubation at pH 8.6. This is not consistent with the measured lumen pH values of, at most 0.3 pH units to the acid side (Table I).

Figure 1a and b also shows that illuminating thylakoids (with 0.5 μM nig present) prior to and during the acetylation decreased the acetylation level from that observed in the dark, plus nig, to near the level in the dark, minus nig. The lower acetylation level is believed to be caused by protonation of the amines—rendering them less reactive—owing to protons released by the redox reactions. This result is consistent with earlier results (Baker *et al.*, 1981) showing that protons produced by the redox systems can keep the acetylatable amines in the protonated, unreactive form, even when significant concentrations of uncouplers are present. Obviously, rather low amounts of protons can drop the pH in the domains or the lumen down to the pK_a of the amines (near 7.5) from the starting pH near 8.6.

Cytochrome f Acetylation with [^{3}H]Accetic Anhydride

Thylakoids used for the isolation of cyt *f* were derivatized in the dark with [^{3}H]acetic anhydride either with the sequestered proton domains filled or dissipated by nig, and in the light with nig present. As stated in the Materials and Methods section, cyt *f* was extracted from treated thylakoids first as the cyt b_6/f complex, followed by SDS-PAGE. The identity of the cyt *f* band, which ran with a mobility near 33 kDa, was verified by heme staining. Cytochrome *f* was labeled 44–49% more in the + nig (proton-depleted state)

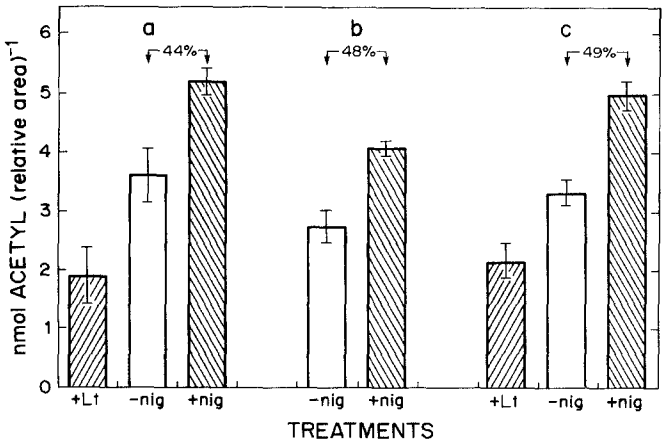

Fig. 3. [^{3}H]acetyl incorporation into cyt *f* isolated from thylakoids which had been acetylated with 0.2 mM [^{3}H]acetic anhydride under complete darkness with (+nig) or without (−nig) 0.5 μM nig or saturating light with 0.5 μM nig (+Lt). All thylakoids were stored in Mes resuspension buffer at pH 6.5 prior to acetylation. In experiments a and c, 3 min, and in experiment b, 5 min, were allowed for the thylakoids to equilibrate in the dark prior to acetylation. All other conditions were as described in the Materials and Methods section. The data represent the average of at least three replicates that were normalized by densitometry as described in Fig. 1.

compared to the −nig treatment (Fig. 3). Chloroplasts illuminated during the acetylation step showed significantly less acetylation compared to the labeling in the dark without uncoupler (Fig. 3a, c).

The pH dependence of cyt *f* acetylation shown in Fig. 4 indicates that if a 0.3 ΔpH (lumen acid) occurred in the absence, but not the presence, of nig, then about a 38% labeling increase might be accounted for by lumen-facing amine groups. This is close to the 44–49% change shown in Fig. 3.

If the cyt b_6/f complex was first solubilized, precipitated by ammonium sulfate, then dialyzed prior to acetylation, there was no significant difference in the labeling of cyt *f* with or without nig (data not shown).

The other three polypeptides of the cyt b_6–*f* complex (cyt b_6, 22 kDa; the Rieske Fe–S protein, 20 kDa; and the 17 kDa subunit) did not show significant acetylation differences between the plus and minus nig treatments(data not shown).

Contribution of Oxygen-Evolving Complex Polypeptides to Sequestered Domain Amine Groups

Because cyt *f* demonstrated a large difference in labeling due to the small ΔpH dissipated by nigericin, we were concerned about the validity of the previous assignment of the extrinsic OEC polypeptides, as contributing amine groups to the sequestered domain (Laszlo *et al.*, 1984b). Repeating

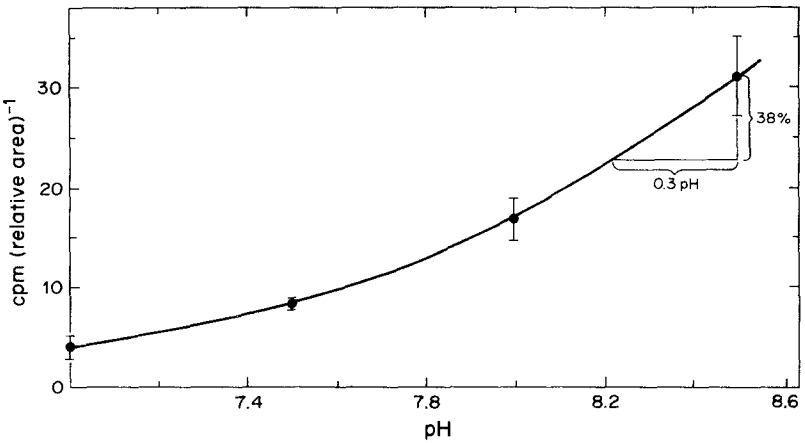

Fig. 4. Labeling of partially purified cyt *f* with [^{3}H]acetic anhydride as a function of the pH. Acetylation was performed in buffer containing 50 mM each of TAPS, MOPS, and KCl and 0.5% cholic acid with 250 μg protein per ml. All other conditions were as described in the Materials and Methods section. The data represent the average of two experiments which were normalized by densitometry as described in Fig. 1. The top and bottom of the error bars give the sample standard deviation.

Table II. [^{3}H]Acetic Anhydride Labeling of the 33, 24, and 18 kDa Polypeptides of the Oxygen-Evolving Complex in Dark-Maintained Thylakoids in the Presence or Absence of 0.5 μM Nigericin[a]

Polypeptide mol. wt. (kDa)	Relative specific activity (cpm/rel. area)		Percent labeling difference $\frac{+\text{nig} - (-\text{nig})}{-\text{nig}} \times 100$
	− nig	+ nig	
33	91 ± 11	180 ± 13	97%
24	42 ± 4	87 ± 7	107%
18	54 ± 5	93 ± 8	72%

[a] Isolated thylakoids were treated with 0.2 mM [^{3}H]acetic anhydride at pH 8.6 in the dark, and the OEC polypeptides were purified and assayed for radioactivity as described in the Materials and Methods section. The sammple standard deviations shown were calculated from the results of three SDS-PAGE gel separations of proteins from one experiment. Experiments conducted on other thylakoids samples gave similar results (data not shown).

those experiments using MES buffer in the thylakoid storage buffer and allowing a 3 min equilibration prior to acetic anhydride addition gave uncoupler-induced labeling increases between 72–107% (Table II), in excellent agreement with the earlier results. The pH dependence of acetylation for the three polypeptides was determined, as shown in Fig. 5. The data show that a ΔpH of 0.3 would contribute about a 25% increase in labeling for a ΔpH between 8.3 and 8.6 (Table III). These data support the previous suggestion that

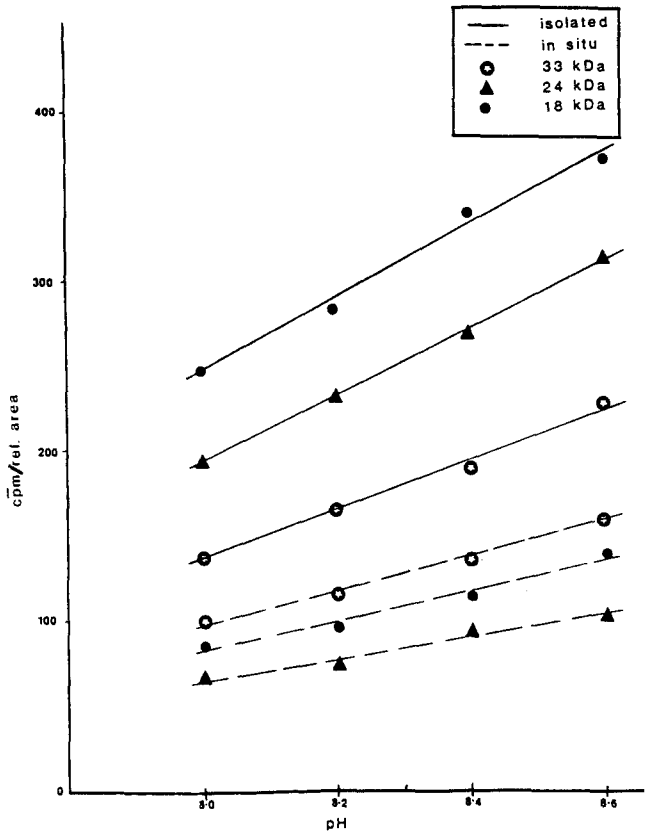

Fig. 5. pH dependence of [^{3}H]acetic anhydride acetylation of the three extrinsic OEC polypeptides. *In situ* labeling: Thylakoids were prepared and stored in the pH 6.5 MES-based buffer described in the Materials and Methods section. Treatment with 0.2 mM [^{3}H]acetic anhydride was as described in the Materials and Methods section at pH values of 8.0, 8.2, 8.4, and 8.6. Nigericin, 0.5 μM and 0.5 μM valinomycin were added 30 sec prior to the acetylating reagent to assure that complete pH equilibration occurred before acetylation commenced. The extrinsic OEC polypeptides were isolated from PSII particles prepared by the method of Berthold *et al.* (1981) from the [^{3}H]acetylated thylakoid membranes. SDS-PAGE gels were run on the isolated polypeptides, and radioactivity in the respective bands was determined as described in the Materials and Methods section. Isolated protein labeling: Acetylation of the isolated OEC extrinsic polypeptides was carried out in a reaction medium similar to that used for thylakoids mentioned above except no uncouplers were added. The three proteins in question were released from the PSII particles by the $CaCl_2$ wash method (Ono and Inoue, 1983), dialyzed against 5 mM Mes, pH 6.5, concentrated by lyophilization, and resuspended as a concentrated protein solution in pH 6.5 MES buffer similar to that used for storing thylakoids. After protein determination, aliquots were diluted into acetylation buffer (as described in Materials and Methods, but no uncoupler was added) at the four pH values, and acetylated with 0.2 mM [^{3}H]acetic anhydride. The acetylated polypeptides were prepared for SDS-PAGE separation and radioactivity determined in the three bands as described in Materials and Methods.

Table III. Acetic Anhydride Incorporation into the Oxygen-Evolving Complex Extrinsic Polypeptides Due to a 0.3 ΔpH Unit Difference[a]

Condition of peptides	Percent labeling difference (pH 8.3–8.6)		
	33 kDa	24 kDa	18 kDa
In situ	24 ± 1	23 ± 3	23 ± 2
Isolated	25 ± 1	23 ± 2	21 ± 1

[a] *In situ* acetylation of isolated thylakoid membranes with [^{3}H]acetic anhydride was performed at 22°C on thylakoids in the presence of 5 μM nig and 0.5 μM valinomycin, as described in the Materials and Methods section and in the legend to Fig. 5. Labeling of isolated polypeptides was performed as described in the legend to Fig. 5 at 20°C with no added protonophores. The percent labeling differences were determined (± standard deviation) from plots shown in Fig. 5 of label associated with the peptide versus pH, comparing labeling at pH 8.3 to 8.6: [ΔCPM(pH 8.6 − pH 8.3)/CPM pH 8.3] × 100. The data to determine the pH dependence of the acetylation utilized pH values of 8.0, 8.2, 8.4, and 8.6, as shown in Fig. 5.

the extrinsic OEC polypeptides contribute significantly to the amine groups of thylakoid sequestered buffering domains.

Acetylation of Stroma-Facing Thylakoid Proteins

The α and β subunits of the CF_1 complex face the stroma and their acetylation pattern was determined in the same manner as described above. Figure 6 shows that no differences occurred in labeling in the α and β subunits either before or after nig addition, or when the thylakoids were illuminated under electron transport conditions prior to and during the acetylation.

Discussion

The quantitative assay used herein for membrane domain-sequestered amine groups depends on establishing conditions where the thylakoid lumen reaches a pH at or measurably near the external value without disturbing the metastable proton pool. Only under these conditions can the uncoupler-induced differential acetic anhydride labeling be an accurate indicator of the buried, metastably protonated amine groups. The metastable domains slowly lose protons to the pH 8.6 suspending phase (Laszlo *et al.*, 1984a), so equilibration times of tens of minutes are counterproductive. Our compromise was to use equilibration times of 3 or 5 min and determine the lumen pH attained in that time, then make a correction to our data for the residual ΔpH-dependent labeling. Either 3 or 5 min incubation times at pH 8.6 resulted in the lumen attaining a pH near 8.3–8.4, measured by [^{14}C]methylamine distribution (Table I). By measuring the pH dependence of acetylation of the

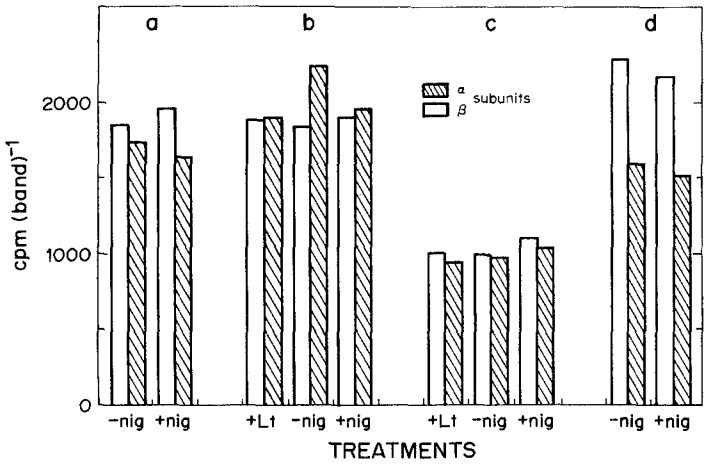

Fig. 6. Incorporation of [^{3}H]acetyl into the α and β subunits of CF_1 from thylakoids which were previously acetylated with 0.2 mM [^{3}H]acetic anhydride in the presence or absence of 0.5 μM nigericin. In experiments a and b thylakoids were stored in Hepes, pH 7.5, resuspension buffer with 3 min equilibration, and in experiments c and d the thylakoids were stored in Mes, pH 6.5, resuspension buffer with a 5-min equilibration in the dark prior to acetylation. All other conditions were as described in the Materials and Methods section. Data are the average of three replicates and are normalized to protein concentration.

thylakoid proteins in question, we could test whether the uncoupler-induced proton release from the putative sequestered domains *in situ* resulted in differential acetylation greater than that estimated for a residual ΔpH of 0.3.

Thylakoid proteins previously shown to provide amine groups to the sequestered domain are the 8 kDa subunit of the CF_0 (Tandy *et al.*, 1982), the three extrinsic OEC polypeptides, and LHC polypeptides (Laszlo *et al.*, 1984b). The present results support the assignment of the three Tris-releasable photosystem II proteins as contributors of sequestered amine groups. When the estimated 0.3 pH unit correction is used, the three polypeptides show considerable additional uncoupler-dependent acetylation (Fig. 5 and Tables II and III).

Surprisingly, PC may also contribute an amine group(s) to the sequestered domains. Plastocyanin has been suggested to be, at least in some cases, a mobile redox component in the lumen (Haehnel, 1984); therefore we did not expect it to contain domain-sequestered amine groups. However, the present results call for a re-evaluation of this view and suggest that PC may bind tightly to its functional site, and when so bound have a region that is buried in the membrane. Haehnel's studies of electron transport through the

PC pool (Haehnel, 1984) were consistent with the protein—during redox turnovers—being tightly bound to the thylakoid in a ternary complex between cyt *f*–PC–P700 rather than functioning as a freely diffusible electron carrier. The results of Anderson *et al.* (1987) also suggested such a ternary complex based on chemical modification of PC carboxyl groups. Moreover, those authors suggested that the cyt *f* binding interaction is with the carboxyls 42–45 and 59–61 of PC with the PS I complex binding through the carboxyls 68 and 59–61 of PC, such that electron transport to P700 proceeds via the His 87 residue. Those speculations need to be substantiated, but they provide a starting point for the purposes of considering possible orientations of lysine residues that could be involved in the "buried" amine group array implicated by our results. Spinach PC has 6 lysine residues (Katoh *et al.*, 1962), five of them located in the "bottom" one-third of the molecule, when viewing it from the perspective—provided from the X-ray crystallographic work of Colman *et al.* (1978)—with the Cu site near the top and the four carboxylic group residues (Nos. 42–45) protruding on the lower right of the structure. Some of the lysines may be in close juxtaposition to positive charges [to induce the low pK_a values that favors rapid reaction with acetic anhydride (Dilley *et al.*, 1987)] and be forced by the ternary complex into an orientation which sequesters the lysines away from direct exposure to the lumen. Future experiments using the present techniques combined with isolating fragments of the PC sequence could identify which lysine residues show the anhydride reactivity predicted for a low pK_a (buried) lysine.

That the structural arrangement of PC in the thylakoid may be more, rather than less, tightly associated with a cleft or binding site on the lumen side of the thylakoid membrane is consistent with other results as well. For example, PC was more highly labeled than expected using [^{35}S]diazobenzene sulfonate, a reagent considered as quite impermeable across the membrane (Smith *et al.*, 1977). Those results were taken as evidence for PC being deeply embedded in the thylakoid such that the reagent could reach at least part of the protein. Also, polylysine apparently interacts with PC, which would not be expected if the protein were completely immersed in the lumen (Brand *et al.*, 1972). Results with PC antibodies suggested that in some circumstances the antibody could reach an antigenic epitope of PC (Boehme, 1978). However, other antibody work consistently obtained contradictory results (Haehnel *et al.*, 1981).

The only completely stromal-facing proteins probed, the α and β subunits of the CF_1, were not differentially acetylated under the conditions used for these studies (Fig. 6). This indicates that the uncoupler-induced acetylation differences detected in the various proteins reported herein are, as

we assumed at the outset of this work, reasonably interpreted as owing to variable protonation states influenced by the uncoupler-induced proton release from behind the membrane diffusion barrier.

Cytochrome *f*, previously suggested as contributing to the array of buried amine groups (Dilley *et al.*, 1987), does not show a labeling pattern justifying that assignment. Similarly, the other polypeptides of the cyt b_6/f complex apparently do not contribute to the buried lysine array (data not shown). The $\approx 47\%$ acetylation increase followed nigericin addition was only marginally larger than the $\approx 38\%$ acetylation difference expected for a 0.3 ΔpH change in the lumen following nigericin addition (Figs. 3 and 4). The majority of the reactive amine groups in cyt *f* detected by this assay are logically assigned as facing the lumen. However, other acid–base dissociation groups of cyt *f* may contribute to the sequestered domain and participate in the putative proton relay mechanism hypothesized to function as the mechanism for protons diffusing in localized domains (Dilley *et al.*, 1987).

The identification of numerous lumenal-facing thylakoid proteins, including the OEC extrinsic polypeptides and the 8 kDa CF_0 subunit, as containing differentially labeled amine buffering groups lends support to the idea that the sequestered proton buffering pool may be provided by proteins with significant portions of their mass located on the lumen side of the membrane. One model we are currently testing is that the bulky parts of lumen-exposed, but membrane-interacting, proteins may provide a type of sequestered space between the lipid bilayer and the protein mass protruding into the lumen, such as to obstruct free H^+ diffusion into the lumen. Other models may better explain the puzzling phenomena of localized proton diffusion, but for the moment, our present model, shown in Fig. 4 of Dilley *et al.*, 1987 (for a model incorporating recent developments, see Fig. 3 of Chiang and Dilley, 1987), is one that guides our hypothesis testing. The present data are consistent with the concept embodied in the model, but clearly we need further structural data to elucidate the physical structure of the localized proton pathway. In spite of the uncertainties about the putative localized domain structures, it is worthwhile to pursue such studies because of the growing body of data from functional (energy coupling) experiments which support the concept that thylakoids can develop either a delocalized or localized protonmotive force sufficient to drive ATP formation (Beard and Dilley, 1988; Graan *et al.*, 1981; Horner and Moudrianakis, 1983; Pick *et al.*, 1987; Sigalat *et al.*, 1985). Other evidence suggests that the sequestered proton buffering domains provide the structures through which the localized energy coupling proton flux occurs (Theg *et al.*, 1988). Thus, as more structural and functional information becomes available, we should be able to test more critically various hypotheses for membrane-organelle energy-coupling mechanisms.

Acknowledgments

This work was supported in part by a grant from the National Science Foundation. The authors thank Janet Hollister for excellent assistance in manuscript preparation.

References

Anderson, G. P., Sanderson, D. G., Lee, C. H., Durell, S., Anderson, L. B., and Gross, E. L. (1987) *Biochim. Biophys. Acta* **894**, 386–398.
Baker, G. M., Bhatnagar, D., and Dilley, R. A. (1981) *Biochemistry* **20**, 2307–2315.
Baker, G. M., Bhatnagar, D., and Dilley, R. A. (1982) *J.. Bioenerg. Biomembr.* **14**, 249–264.
Beard, W. A., and Dilley, R. A. (1988) *J. Bioenerg. Biomembr.* **20**, 129–154.
Beard, W. A., Chiang, G., and Dilley, R. A. (1988) *J. Bioenerg. Biomembr.* **20**, 107–128.
Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1981) *FEBS Lett.* **134**, 231–234.
Black, M. T., Widger, W. R., and Cramer, W. A. (1987) *Arch. Biochem. Biophys.* **252**, 655–661.
Boehme, H. (1978) *Eur. J. Biochem.* **84**, 87–93.
Brand, J., Baszynski, T., Crane, F. L., and Krogmann, D. W. (1972) *J. Biol. Chem.* **247**, 2814–2819.
Chiang, G., and Dilley, R. A. (1987) *Biochemistry* **26**, 4911–4916.
Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., and Venkatappa, M. P. (1978) *Nature (London)* **272**, 319–324.
Dilley, R. A., and Schreiber, U. (1984) *J. Bioenerg. Biomembr.* **16**, 173–193.
Dilley, R. A., Theg, S. M., and Beard, W. A. (1987) *Annu. Rev. Plant Physiol.* **38**, 347–389.
Ellefson, W. L., Ulrich, E. A., and Krogmann, D. W. (1980) *Methods Enzymol.* **69c**, 223–228.
Graan, T., Flores, S., and Ort, D. R. (1981) In *Energy Coupling in Photosynthesis* (Selman, B. R., and Selman-Reimer, S., eds.), Elsevier/North Holland, New York, pp. 25–34.
Haehnel, W. (1984) *Annu. Rev. Plant Physiol.* **35**, 659–693.
Haehnel, W., Berzborn, R. J., and Andersson, B. (1981) *Biochim. Biophys. Acta* **637**, 389–399.
Hoober, J. K. (1970) *J. Biol. Chem.* **245**, 4327–4334.
Horner, R. D., and Moudrianakis, E. N. (1983) *J. Biol. Chem.* **258**, 11643–11647.
Kamienietzky, A., and Nelson, N. (1975) *Plant Physiol.* **55**, 282–287.
Katoh, S., Shiratori, I., and Takamiya, A. (1962) *J. Biochem.* **51**, 32–40.
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
Laszlo, J. A., Baker, G. M., and Dilley, R. A. (1984a) *J. Bioenerg. Biomembr.* **16**, 37–51.
Laszlo, J. A., Baker, G. M., and Dilley, R. A. (1984b) *Biochim. Biophys. Acta* **764**, 160–169.
Ono, T., and Inoue, Y. (1983) *FEBS Lett.* **164**, 255–260.
Ort, D. R., and Izawa, S. (1973) *Plant Physiol.* **52**, 595–600.
Pfister, V. R., and Homann, P. H. (1986) *Arch. Biochem. Biophys.* **246**, 525–530.
Pick, U., Weiss, M., and Rottenberg, H. (1987) *Biochemistry* **26**, 8295–8302.
Rottenberg, H., Grunwald, T. and Avron, M. (1972) *Eur. J. Biochem.* **25**, 64–70.
Sigalat, C., Haraux, F., de Kouchkovsky, F., Hung, S. P. N., and de Kouchkovsky, Y. (1985) *Biochim. Biophys. Acta* **809**, 403–413.
Smith, D. D., Selman, B. R., Voegli, K. K., Johnson, G. and Dilley, R. A. (1977) *Biochim. Biophys. Acta* **459**, 468–482.
Tandy, N. E., Dilley, R. A., Hermodson, M. A., and Bhatnagar, D. (1982) *J. Biol. Chem.* **257**, 4310–4307.
Theg, S. M., Johnson, J. D., and Homann, P. H. (1982) *FEBS Lett.* **145**, 25–29.
Theg, S. M., Chiang, G. G., and Dilley, R. A. (1988) *J. Biol. Chem.* **263**, 673–681.
Thomas, P. E., Ryan, D., and Levin, W. (1976) *Ann. Biochem.* **75**, 168–176.