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CHLOROPLAST THYLAKOID MEMBRANE PROTEINS HAVING BURIED AMINE BUFFERING GROUPS

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Some chloroplast thylakoid membrane proteins have anomalously low pK_a (near 7.8) amine groups, indicating that the buffering groups may be buried in hydrophobic regions and/or close to other positive charges. Other work has shown that the low pK_a amine group array is not in ready equilibrium with either the inner or outer bulk aqueous phases (Laszlo, J.A., Baker, G.M. and Dilley, R.A. (1984) J. Bioenerg. Biomembranes, 16, 37–51). Acetic anhydride reacts with the neutral amine and has been used as a probe for labeling the low pK_a amines. The buried array of buffering groups can be labeled with [^{3}H]acetic anhydride in the dark only after the membranes were made leaky to protons with uncoupler addition. Sodium dodecyl sulfate/urea-polyacrylamide gel electrophoresis was used to separate the polypeptides and identify those that show the uncoupler-dependent labeling increase. Included in that group are polypeptides known to be associated with Photosystem II having M_r 17000, 22000 and 31000, some of the light-harvesting pigment proteins with M_r 24000–28000, the CF_0 component with M_r 8000 and some polypeptides associated with Photosystem I. A protein with M_r 15000 showed very large changes in labeling, but the identity of this polypeptide is unknown. The arrays of buried amine buffering groups are diversely distributed among membrane proteins and it is not clear what role, if any, they play in membrane function.

Introduction

Considerable evidence in membrane bioenergetics research suggests that, in support of the basic tenents of the Mitchell hypothesis [1,2], a transmembrane, bulk phase-to-bulk phase, electrochemical potential, or proton motive force, is necessary and sufficient to drive either oxidative phosphorylation or photophosphorylation (see Ref. 3 for a review). However, there are many observations suggesting that proton movements linked to energy transduction occur primarily through membrane-associated channels, while proton movements to the aqueous phases are of secondary consequence [4–13], in keeping with Williams' concept of the proton motive force-driven reaction [14]. Various membrane structures that may support proton movements along the surface of, or through, membranes have been suggested [5,15–17]. Considering the mobility of protons and the complexity of the membrane structure, it is not surprising that direct measurements of proton movements in localized membrane domains are lacking.

We have used acetic anhydride as a chemical modification probe for membrane-protein interac-

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Abbreviations: Chl, chlorophyll; LHC, light-harvesting chlorophyll *a*/*b*-protein complex; PS, photosystem; CF_0-CF_1, ATP synthetase complex; Hepps, *N*-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

tions with protons [4,18–23]. Acetic anhydride reacts selectively with unprotonated primary amine groups, such as lysine residues. Utilizing this technique, we have demonstrated that dark-maintained chloroplast thylakoid membranes retain a pool of protons which do not readily equilibrate with either the inner or outer bulk aqueous phases [22]. The membrane-bound proton buffering pool displays the following characteristics: (1) The 'special pool' of protons, 30–40 nmol · (mg Chl^{-1}), are behind the permeability barrier of the membrane, apparently bound to an array of amine buffering groups in a metastable state [20]. In dark-adapted membranes this buffering group array is usually in the protonated, anhydride-unreactive state. Uncouplers or a brief thermal treatment cause the release of the bound proton pool into a pH 8.5 suspension medium, directly detectable with a pH-sensitive electrode or indirectly by increased acetic anhydride modification of the membrane proteins [4,20]. Homann and colleagues [24,25] have independently reported on uncoupler-induced proton efflux in dark-adapted chloroplasts, showing that under some conditions the amount of proton efflux can greatly exceed 30–40 nmol H^+ ·(mg $Chl)^{-1}$. (2) Either electron transfer-linked proton accumulation [20] or ATPase proton pumping [21] converts the uncoupler or thermal treatment-induced deprotonated state to the protonated, anhydride-resistant state. (3) A subset of the acetic anhydride-reactive groups is associated with the water oxidizing apparatus. That subset of amine groups has an apparent pK_a of 7.8 [22]. It is also known that the buffering array includes the lysine 48 residue of the M_r 8000 CF_0 component [19,23]. (4) The bound protons, in part at least, are retained by dark-maintained membranes equilibrated with pH 8.8 buffer for at least 1 h in the absence of a protonophore, even though the inner, bulk aqueous phase also reaches a pH near 8.7 [22]. (5) The lag in the onset time of ATP formation in flashing light is closely correlated with the protonation state of the amine buffering array. Thylakoids in the unprotonated state display a longer lag than when starting in the protonated state, and the effect is reversible [26]. The number of flashes involved in that effect corresponds to about 30 nmol H^+·(mg $Chl)^{-1}$, close to the size of the amine buffering pool.

The above characteristics of the 'special proton pool' are consistent with the hypothesis that the amine group array may be involved in the mechanism of proton movement along localized domains of the membrane, from proton release sites into the CF_0-CF_1 proton sinks. Part of the data base necessary to evaluate the role of the amine buffering array is knowledge as to which membrane proteins provide the amine groups constituting the array. In this work we have used the acetic anhydride probe to identify those membrane proteins.

Materials and Methods

Chloroplast isolation, protein assay, and electron transport activity assay. Chloroplasts were isolated from spinach following the method of Ort and Izawa [27]. Chlorophyll concentration was determined by the method of Arnon [28], and protein determined as described in Ref. 19. Electron transport activities (water to methyl viologen) were measured as described in Ref. 20.

Acetic anhydride modification. Derivatization of membrane proteins with 3.5 mM acetic anhydride was as described in Refs. 20 and 22. For radiolabel experiments, [^{3}H]acetic anhydride (Amersham Corporation) was used at 0.2 mM (either 500 or 125 Ci/mol), except where noted otherwise. Chloroplast membranes were suspended at 20°C in 50 mM Hepps-NaOH (pH 8.6) containing 50 mM KCl, 2 mM $MgCl_2$, 0.1 M sucrose and, when present, either 0.5 or 0.05 μM nigericin. The Chl concentration was 40 μg/ml for all experiments except those which required the preparation of PS II particles. For the latter experiments, the Chl concentration was 50 μg/ml. Acetic anhydride modification was initiated after either a 30 s dark or 15 s illumination period (with 0.1 mM methyl viologen present). After 30 s, the reaction was quenched by the addition of 50 mM *N*-glycylglycine (pH 8.6).

[^{3}H]Acetic anhydride-derivatized membranes were partially delipidated with 80% acetone, suspended in 5% SDS and an aliquot (10–40 μl) of the sample was added to 10 ml of Tritosol scintillation cocktail [29] for counting.

Preparation of PS II particles and Tris-released polypeptides. Photosystem II particles were pre-

pared from acetic anhydride-derivatized membranes using a procedure adopted from Yamamoto et al. [30,31]. Chloroplasts were suspended at 1 mg Chl/ml in 20 mM Mes-NaOH (pH 6.5) containing 5.0 mM $MgCl_2$ and 0.33 M sorbitol. Digitonin and Triton X-100 were added to final concentrations of 0.25% (w/v) and 0.20% (w/v), respectively. The suspension was stirred for 5 min on ice, diluted 1:10 with the above buffer and centrifuged at $12000 \times g$ for 20 min. The pellet was resuspended in a small volume of the same buffer.

The Tris-releasable polypeptides were obtained by incubating the PS II particles in 0.8 M Tris-HCl (pH 8.9) on ice for 20 min, then centrifuging the suspension as above. The supernatant was concentrated by diafiltration using a 10 ml Amicon ultrafiltration cell equipped with a YM 10 filter. The supernatant peptides were precipitated with 3% trichloroacetic acid and resuspended in a small volume of 5% SDS.

Electrophoretic analysis. SDS-polyacrylamide gel electrophoresis of partially delipidated chloroplast proteins was carried out on 15% (w/v) acrylamide/0.2% (w/v) *N,N*-methylenebisacrylamide slab gels containing 6 M urea, utilizing the conditions described in Ref. 32. In our hands, resolution of polypeptides on the straight 15% polyacrylamide gels was equal or superior to that of the 12.5–25% polyacrylamide gradient gels used formerly [32]. Assignment of particular molecular weights to individual polypeptides was as given in [32].

For characterization of chlorophyll-protein complexes, derivatized thylakoid membranes were solubilized in 2.0% SDS (SDS:Chl = 20) for 15 min, then applied immediately to a 10% polyacrylamide gel (containing 0.1% SDS but lacking urea) and electrophoresed for 5 h at 4°C.

Analysis of the [^{3}H]acetyl incorporation into membrane peptides following electrophoresis was achieved by slicing the gel into 1.78 mm segments, followed by digesting the slices and scintillation counting as described in Ref. 32.

Results

Effects of acetic anhydride modification on the electrophoretic mobility of thylakoid membrane proteins

Previously, we have used about 3.5 mM acetic anhydride to determine the status (i.e., whether protonated or unprotonated) of amine groups associated with membrane proteins [20–22]. Derivatization of thylakoids with that concentration of acetic anhydride apparently altered the electrophoretic mobility of some of the chloroplasts proteins (Fig. 1). In particular, three polypeptides with relative molecular masses of 31000, 22000, and 17000, appeared to be missing completely in the gel scan when the chloroplasts were modified in the dark with uncoupler present (Fig. 1B), conditions giving maximal [^{3}H]acetyl incorporation

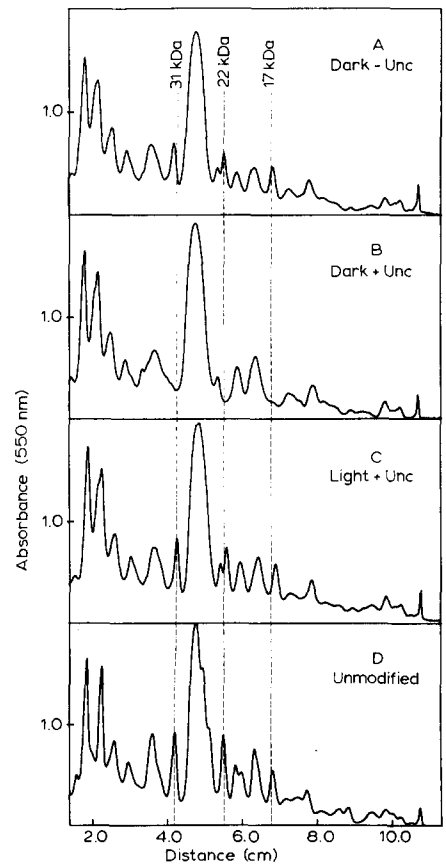

Fig. 1. Gel scans of thylakoid membrane proteins. Thylakoid membranes were treated with 3.5 mM acetic anhydride (A) in the dark in the absence of uncoupler, (B) in the dark in the presence of 0.5 μM nigericin, (C) in the light in the presence of 0.05 μM nigericin, (D) and in the presence of 50 mM *N*-glycylglycine (effectively unmodified). Equivalent amounts (160 μg) of each sample were loaded on the SDS/urea-polyacrylamide slab gel.

into thylakoid membrane proteins. That result makes it difficult to use the SDS-polyacrylamide gel electrophoresis system to quantitate the state of amine group protonation of membrane proteins after various treatments. Conditions had to be found to allow probing the protonation state without altering the mobility or staining characteristics of the proteins. This was accomplished by lowering the concentration of acetic anhydride.

Using 0.2 mM acetic anhydride did not affect the electrophoretic mobility of the proteins (Fig. 2). The lower concentration of acetic anhydride gave similar plus versus minus uncoupler incorporation differences as the 3.5 mM acetic anhydride. The average difference in incorporation was 19–20% with either 0.2 mM or 3.5 mM acetic anhydride (Table I). Illumination of an uncoupler-treated thylakoid sample during the derivatization with 0.2 mM acetic anhydride, as with 3.5 mM acetic anhydride, resulted in a decreased level of derivatization (data not shown). So, although lower concentrations of acetic anhydride did not affect electron transport activity (i.e. no inhibition in the dark in the presence of uncoupler), as did the 3.5 mM acetic anhydride (data not shown), use of the 0.2 mM acetic anhydride as a probe for the sites of proton-protein interaction seems justified.

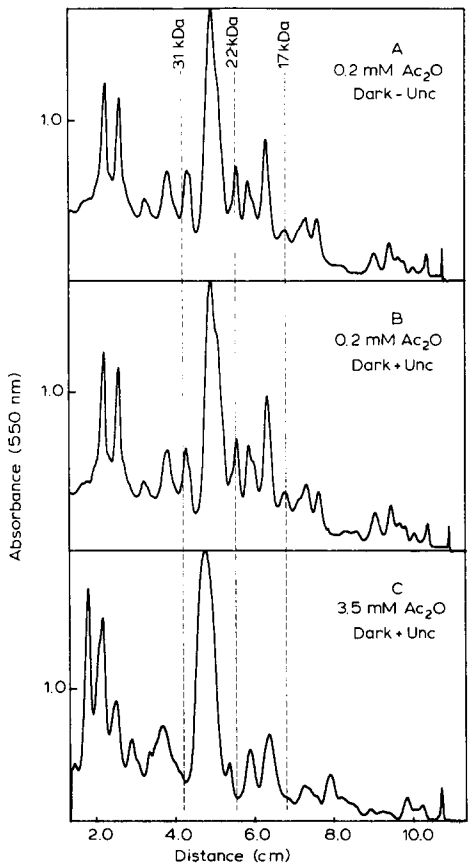

Fig. 2. Gel scans of acetylated thylakoid membrane proteins. Thylakoid membranes were treated with (A) 0.2 mM acetic anhydride in the dark in the absence of uncoupler, (B) 0.2 mM acetic anhydride in the dark in the presence of 0.5 μM nigericin, (C) and 3.5 mM acetic anhydride in the dark in the presence of 0.5 μM nigericin. The scans in panels A and B were from a different SDS/urea-polyacrylamide slab gel than panel C. Panel C is the same scan as in Fig. 1, B. Therefore, the polypeptide bands do not exactly coincide between the top two and bottom panels.

TABLE I

INCORPORATION OF [^{3}H]ACETYL INTO DARK-MAINTAINED THYLAKOID MEMBRANE PROTEINS

Thylakoid membranes were treated with 0.2 mM [^{3}H]acetic anhydride (125 Ci/mol) in the dark, with and without 0.5 μM nigericin present. 50 μg each of the control (−nigericin) and plus nigericin samples were counted. The counting efficiency was 40%. The percentage difference was calculated as $((cpm_{+u} - cpm_{-u})/cpm_{+u}) \times 100$.

pH	Radioactivity of membrane proteins (cpm/μg)		% Difference
	−Nigericin	+Nigericin	
0.2 mM acetic anhydride			
8.6	2580	3190	19
8.0	1670	2080	20
3.5 mM acetic anhydride (data from Fig. 2)			
8.6	290	365	20
8.0	210	260	19

Incorporation of acetic anhydride into thylakoid membrane proteins

Fig. 3 shows data from SDS/urea-polyacrylamide gel electrophoresis gels of the incorporation of [^{3}H]acetyl into thylakoid membrane proteins, modified in the dark in the presence and absence of uncoupler. The values presented span the region from the α and β subunits

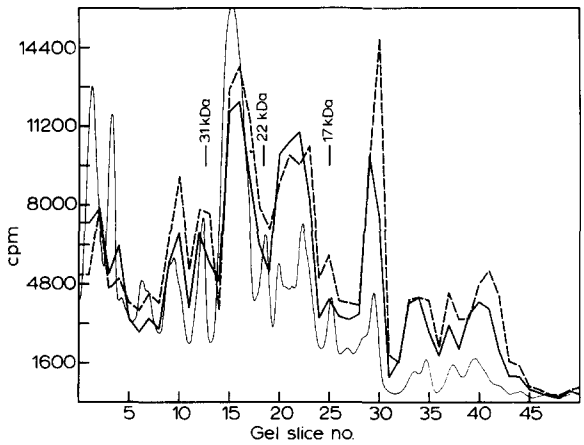

Fig. 3. Protein labeling at low concentrations of acetic anhydride. Thylakoid membranes were treated in the dark with 0.2 mM [^{3}H]acetic anhydride (500 Ci/mol) either in the presence (dashed line) or the absence (heavy solid line) of 0.5 μM nigericin. A representative gel scan of the membrane proteins is superimposed (light solid line) to illustrate the coincidence of polypeptides with specific gel slices. The top approx. 1.5 cm of the gel, being essentially devoid of discernible bands, was neither counted nor included in the gel scan. Exactly 160 μg of each sample, a total of $1.28 \cdot 10^6$ and $1.03 \cdot 10^6$ dpm of the plus nigericin and the no nigericin samples, respectively, were loaded on each lane. The counting efficiency of the gel slices was 28%.

of CF_1 (gel slices 1–4) to the M_r 6000–8000 region (gel slices 40–45). Replicate gel lanes of a given sample generally showed less than 5% variation between comparable gel slices. Labeling differences due to the treatment – i.e., dark plus uncoupler versus dark minus uncoupler – occurred in quite a few polypeptides. Such regions include: M_r 35000 (gel slices 10 and 11), 31000 (gel slice 13), LHC (gel slices 16–18), 22000 (gel slice 20), 17000 (gel slice 26), 15000 (gel slice 30), and 8000 (gel slices 42 and 43). The large incorporation differences in the M_r 31000, 22000, 17000 polypeptides probably accounts for the altered mobilities of these three polypeptides when modified with 3.5 mM acetic anhydride (Fig. 1). The incorporation difference in the M_r 8000 region is due, at least in part, to the presence of the CF_0 proteolipid [19]. Essentially the same labeling pattern is seen when the membranes have been washed with NaBr to remove the CF_1 [33] following derivatization (data not shown). This indicates that the CF_1 polypeptides do not contribute significantly to the incorporation differences. The M_r 15000 polypeptide appeared to be exceptionally heavily labeled in comparison to the other membrane polypeptides, particularly when compared to those of similar staining intensity (compare the M_r 17000 and 15000 bands). As indicated in the preliminary report [19] the M_r 8000 and 15000 regions showed the greatest total labeling and light-dark or dark, $\pm$ uncoupler labeling differences when compared on a relative specific activity basis (i.e., cpm per area of stained band).

A similar analysis of the [^{3}H]acetyl incorporation differences between membranes modified in the dark, in the presence and absence of uncoupler, at pH 8.0, showed essentially the same differences as membranes modified at pH 8.6 (data not shown).

Differential incorporation of [^{3}H]acetyl into Tris-releaseable PS II polypeptides and PS II particles

The three polypeptides (M_r 31000, 22000, and 17000) which showed large mobility changes upon acetylation (Fig. 1) and significant acetylation differences (Fig. 3) probably are the three Tris-releasable polypeptides isolated from either PS II

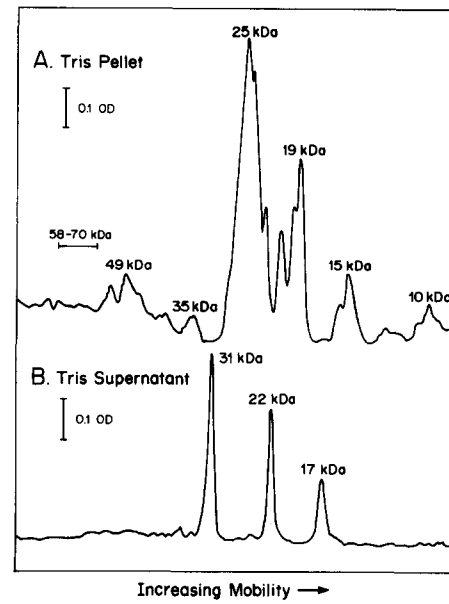

Fig. 4. Gel scans of (A) Tris-extracted PS II particles and of (B) proteins extracted from PS II particles by Tris-extraction. The PS II particles and Tris-extracted proteins were prepared as described in the Materials and Methods section.

particles [30,31,34] or inverted thylakoid membranes [35–37]. This inference was confirmed by isolating PS II particles and treating them with 0.8 M Tris (see Materials and Methods). Three polypeptides with M_r of 31000, 22000, and 17000 were recovered from the Tris-wash supernatant (Fig. 4). When these three isolated polypeptides were acetylated with 5.8 mM acetic anhydride their electrophoretic mobilities were noticeably decreased and the peaks broadened (data not shown).

The Tris-extracted polypeptides isolated from thylakoid membranes treated with 0.2 mM [^{3}H]acetic anhydride in the presence or absence of uncoupler, displayed large differences (approx. 50%, Table II) in acetylation. This incorporation difference exceeded the extent of the labeling differences (percentage difference) of the entire membrane (cf. Table I). Illumination of a plus uncoupler (0.05 μM nigericin) sample prior to anhydride addition led to a further decrease of labeling (Table II). In the light, with 0.05 μM nigericin present, we have measured a proton uptake near 160 μmol · (mg Chl)$^{-1}$ [38]. Hence, the light effect is believed to result from an increased number of protonated low pK_a amine groups.

The PS II particles, after Tris washing, retained peptides that showed labeling differences (Fig. 5). Prominent incorporation differences occurred in the M_r 35000 (gel slices 11 and 12), LHC (gel slices 18–20), M_r 15000 (gel slices 34 and 35), and

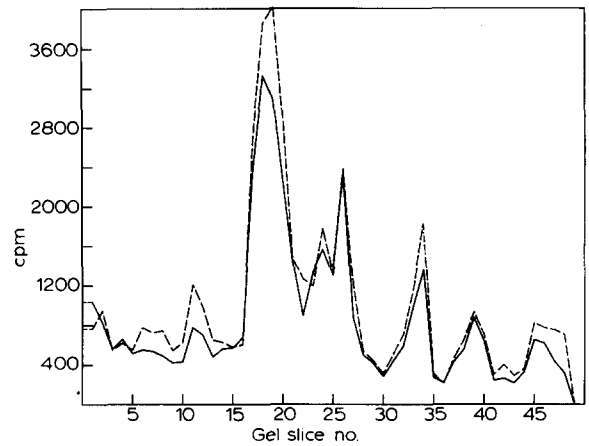

Fig. 5. Differential incorporation of [^{3}H]acetyl into Tris-washed PS II particle-proteins fractionated on a SDS/urea-polyacrylamide slab gel. Tris-washed PS II particles were prepared from thylakoid membranes which had been derivatized with 0.2 mM [^{3}H]acetic anhydride (125 Ci/mol) in the dark either in the presence (dashed line) or absence (solid line) of 0.5 μM nigericin. 160 μg of each sample, $226 \cdot 10^3$ and $193 \cdot 10^3$ dpm of the plus nigericin and minus nigericin samples, respectively, were loaded on each gel lane. The counting efficiency of the gel slices was 28%.

M_r 8000 (gel slices 47–49) polypeptides. Thus, although the labeling differences in the Tris-released polypeptides were large, they by no means constituted all of the proteins showing significant incorporation differences.

TABLE II

INCORPORATION OF [^{3}H]ACETYL INTO THREE TRIS-EXTRACTABLE POLYPEPTIDES

Tris-extractable polypeptides were prepared from [^{3}H]acetic anhydride modified thylakoids as described in the Materials and Methods section. The +nigericin, dark sample had 0.5 μM nigericin, and the +nigericin, light sample had 0.05 μM nigericin. The latter sample, with uncoupler present, was illuminated 15 s before adding the acetic anhydride, with 30 s additional illumination. Approximately 40 μg of each sample were loaded on a SDS/urea gel. After staining with Coomassie brilliant blue G250 and destaining in 10% acetic acid and 10% isopropanol, the area (arbitrary units) under each band was determined with a planimeter [32]. A total of 22400, 40000, and 8000 cpm were loaded of the (−nigericin), the +nigericin, and the light samples, respectively. The specific activity of the [^{3}H]acetic anhydride was 125 Ci/mol (0.2 mM). Percentage differences were calculated as in Table I.

Polypeptide ($M_r \cdot 10^{-3}$)	Relative specific activity (cpm/unit area)			% Difference	
	Dark −nigericin	Dark +nigericin (0.5 μM)	Light +nigericin (0.05 μM)	Comparing +nigericin to −nigericin (Dark)	Comparing +nigericin to +light
31	65.6	138	10.5	52	92
22	75.0	132	5.0	43	96
17	148.0	325	9.0	54	97

Differential incorporation of [^{3}H]acetyl into PS I-associated polypeptides

PS II- and CF_0-associated polypeptides were not the only polypeptides which displayed significant [^{3}H]acetyl incorporation differences. The Chl-protein complexes of the chloroplast membrane may be fractionated on SDS-containing polyacrylamide gels, so-called green gels, under mildly dissociating conditions [39]. The two Chl-protein complexes with the lowest mobilities are those associated with PS I, usually designated CPIa and CPI [39]. The PS I polypeptides(s) showed an [^{3}H]acetyl incorporation difference in the dark, comparing plus and minus uncoupler treatments, or in the light compared to the dark (Table III). The labeling difference in the PS I fraction was similar to that of the whole membrane. Since PS II is thought to occur mostly in the granal portions of the membrane while PS I is believed to be found mainly in the stroma-exposed parts of the lamellae [40–42], this implies that the anhydride-reactive sites are located in both the grana and stroma lamellae.

Time-dependent changes in the differential incorporation of [^{3}H]acetyl

Thylakoid membranes retained their resistance to acetic anhydride inhibition of water oxidation activity for up to 2 h when suspended in high pH (8.6) buffer with no uncoupler and in darkness [24]. Fig. 6 demonstrates the stability of this resistance in membranes suspended in pH 8.6 medium at room temperature. The plus versus minus un-

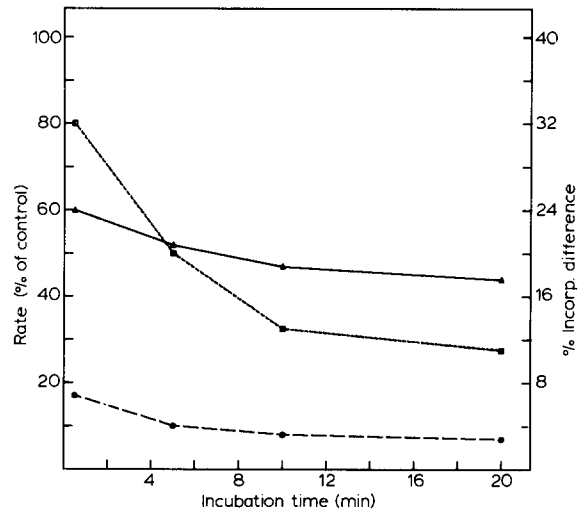

Fig. 6. Time dependence of acetic anhydride inhibition of water oxidation and incorporation of [^{3}H]acetyl from acetic anhydride. Thylakoid membranes were incubated in anhydride reaction buffer (pH 8.6) in the dark at 20°C for varying lengths of time prior to their treatment with acetic anhydride. The electron transport activity (H_2O to methyl viologen) of the samples were measured following derivatization with 3.5 mM acetic anhydride in the presence (○------○) or absence (△———△) of 0.5 μM nigericin and compared to the activity of unmodified samples incubated for the same period of time. The same protocol was employed to determine the differential incorporation of [^{3}H]acetyl as a function of time (□------□) except the membranes were derivatized with 0.2 mM [^{3}H]acetic anhydride (500 Ci/mol). The data are expressed as the percentage difference in incorporation between the dark, no uncoupler, and dark, plus uncoupler, samples. For all of the plus uncoupler cases, the nigericin was added 30 s prior to the anhydride treatment.

TABLE III

INCORPORATION OF [^{3}H]ACETYL INTO Chl-PROTEIN COMPLEXES

Conditions for treating thylakoids with acetic anhydride were as given in Table II. Chl-protein complexes were separated on a SDS-polyacrylamide slab gel, excised, and counted as described in the Materials and Methods section. Equivalent amounts of Chl were loaded on each lane (70 μg). Incorporation into the thylakoid membrane fractions were 3050, 3790, and 2550 cpm/μg Chl for the −nigericin, the +nigericin, and the light samples, respectively. The specific activity of the [^{3}H]acetic anhydride was 125 Ci/mol (0.2 mM).

Chl-protein complex	cpm/Chl-protein complex			% Difference	
	−Nigericin	+Nigericin	Light + nigericin	−Nigericin/+nigericin	Light/+nigericin
CP1a	4460	5430	2890	18	47
CP1	6380	8380	3920	24	53
LHC	29800	34500	17200	14	50

coupler [^{3}H]acetyl incorporation differences, however, decreased over the first 10 min incubation period and then stabilized at about a 12% effect. The slow loss of differential incorporation probably represents the release of protons from their membrane-associated binding sites. The extent of the differential incorporation of [^{3}H]acetyl diminished gradually in all of the polypeptides which showed differences (data not shown).

Discussion

This attempt to determine which thylakoid membrane polypeptide(s) constitute the anomalously low pK_a amine buffering array showed that many polypeptides participate in the uncoupler and light (proton accumulation) modulated acetylation differences. The preliminary light-dark studies [19] using a much lower resolving power SDS-polyacrylamide gel electrophoresis system have been extended with the higher resolution SDS/urea-polyacrylamide gel electrophoresis used here. The main emphasis here was on the acetylation differences observed between the plus and minus uncoupler treatments. To reiterate, the minus uncoupler case is a situation in which a pool of about 30–40 nmol · (mg Chl)$^-$ of potentially acetylatable groups – probably $-NH_3^+$ groups – are not acetylated, even when the lumen aqueous space pH is near 8.7, almost 1 pH unit above the pK_a of the 'special pool' of buffer groups [22]. The model we earlier visualized was that a few proteins may provide the special environment that permits the existence of a sequestered domain, not in ready equilibrium with the two aqueous phases on either side of the membrane. However, the present data suggest that many polypeptides, as defined by SDS/urea-polyacrylamide gel electrophoresis gels, participate in providing the sequestered domain(s). It is not possible to identify the functions of all the polypeptides, nor to say which ones, being discrete bands on the SDS/urea-polyacrylamide gel electrophoresis gels, may in fact be associated as a single oligomeric protein complex in their native state. We can discuss several of the bands in some detail however, and that will follow after some general comments on the techniques used.

It has been shown for both oxygen-evolving PS II particles and inside-out PS II-enriched vesicle preparations [36,37,43], that three polypeptides can be selectively removed from the membranes by extraction with Tris at alkaline pH. That these polypeptides may play a role in oxygen evolution is suggested by the correlation between their extractibility by Tris [31,34] and their trypsin sensitivity [36], and the concomitant inhibition of water oxidation activity. There have been reports [36,37,41] that one, or all three, polypeptides may be active in partially reconstituting water oxidation activity, but contrary reports [44,45] suggested that these polypeptides were not essential for reconstitution. The general concensus is that these three polypeptides are extrinsic membrane proteins located on the inner (lumen) surface of the membrane and are specificially associated with PS II.

The Tris-releasable polypeptides were differentially labeled (Fig. 3 and Table II). The approx. 50% [^{3}H]acetyl incorporation differences between the dark, minus nigericin, and dark, plus nigericin samples of Tris-released polypeptides was greater than the average labeling difference (20%) of the entire membrane extract, indicating that many of what we define as metastable proton-binding sites are on these polypeptides. By comparison, the purified M_r 8000 CF_0 component displays only a 20% incorporation difference between the dark, plus nigericin, case and either the light or dark, minus nigericin, samples [23].

There are about 16, 24 and 30 mol lysyl residues per mol polypeptide for the M_r 17000, 22000 and 31000 Tris-releasable polypeptides, respectively [31]. If we assume one copy each of the three polypeptides per chain and 1.3 nmol chain · (mg Chl)$^{-1}$, then there are about 90 nmol lysine · (mg Chl)$^{-1}$ attributable to the three polypeptides in question. Given that the maximum labeling difference, under our conditions, between plus versus minus uncoupler is around 30–40 nmol · (mg Chl)$^{-1}$ [20], the Tris-releasable peptides only contribute a small proportion of their lysine residues to the special buffering pool.

The Tris-releasable polypeptides are not the only PS II-associated polypeptides with metastable proton binding sites. The polypeptides in the M_r 40000–50000 region of SDS-polyacrylamide gel electrophoresis gels are considered to be components of PS II [46,47]. This region of our SDS/

urea-polyacrylamide gel electrophoresis gels showed [^{3}H]acetyl incorporation differences in both whole thylakoid membranes (Fig. 3, gel slice 7) and Tris-washed PS II membranes (Fig. 5, gel slices 6 and 7). Also, the LHC polypeptides clearly showed significant [^{3}H]acetyl incorporation differences (10–20%), indicating that there are metastable proton binding sites in the LHC (Figs. 3 and 5, Table III). Considering the large amount of LHC in thylakoid membranes (approx. 40% of the total protein [48]), the LHC must contain somewhat less than half of the total metastable proton binding sites.

The data in Table III provide evidence that metastable proton binding sites are not restricted to PS II and CF_0 components entirely, because the PS I Chl-protein complex also displayed significant [^{3}H]acetyl incorporation differences. This is in agreement with our earlier finding that the metastable proton binding sites are accessable, under some conditions, to protons from either PS I- or PS II-driven protolytic events [38]. Apparently the metastable proton binding sites are distributed over several thylakoid polypeptides.

A polypeptide, or group of polypeptides, of M_r 15 000 were exceptionally reactive towards acetic anhydride (Fig. 3, slice 30). The high reactivity suggests that the polypeptide(s) is lysine-rich, like the Tris-releasable polypeptides. There was a large differential [^{3}H]acetyl incorporation difference (plus versus minus nigericine) in this region, indicating that it also contains a large number of metastable proton binding sites. The identity of this polypeptide is unknown. It may be related to the M_r 15 000 polypeptide found by Ellenson et al. [49] to be differentially labeled (light vs. dark) with fluorescamine. Interestingly, they also found polypeptides of M_r 32 000 and 23 000 to be differentially labeled with fluorescamine [49]. They suggested that all three polypeptides were components of the CF_0.

Attempts to identify the water oxidizing enzyme using the acetic anhydride probe have been unsuccessful. The resistance of water oxidation activity to inhibition by acetic anhydride is relatively persistent in membranes suspended in high pH buffer at 20°C for long periods of time; during which time there is a general decline in differential anhydride labeling, suggesting a slow debinding of metastable bound protons (Fig. 6). This suggests that with time the acetic anhydride sensitive amines in water oxidation should represent an increasing proportion of the remaining metastable proton pool. However, when the differential [^{3}H]acetyl incorporation pattern in thylakoid membranes was compared between 0.5 and 20 min incubation periods, no single polypeptide stood out as having an unaltered incorporation difference (data not shown). It is possible that even longer incubation periods in the high pH buffer may be required to show a selective labeling of the water oxidizing polypeptide with the [^{3}H]acetic anhydride probe.

The presence of metastable, sequestered, proton binding sites in thylakoid membranes represents an intriguing phenomenon with relevance to both the fields of bioenergetics and biomembrane structure. The present results indicate that as many as seven to ten polypeptides contribute low pK_a amines to the sequestered domain(s). Among those are proteins associated with at least one of the sources of protons in the redox system (PS II) and the CF_0 energy coupling sink. However, the issue of proton movement along restricted pathways in membranes is an experimentally very difficult topic to deal with. Whether the domain(s) discussed here are involved in such proton processing in bioenergetics (see Introduction) requires further testing. One series of experiments has given evidence consistent with the hypothesis [26].

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