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METASTABLE PROTON POOLS IN THYLAKOIDS AND THEIR IMPORTANCE FOR THE STABILITY OF PHOTOSYSTEM II

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After isolated chloroplast thylakoids have been transferred to a medium which is more alkaline than their storage medium, they retain considerable amounts of unequilibrated protons for often longer than 10 min. Essentially all of these protons are released upon uncoupler addition when the thylakoids are osmotically swollen, but only a portion of them when they are in a shrunken state. Osmotic swelling also greatly accelerates the inactivation of the water-oxidizing system enzyme of Photosystem II, and its depletion of functional Cl^-, at alkaline pH. Analyses of the mestable proton gradient in terms of stoichiometry, temperature dependence, and effect on fluorescent amine probes, suggest that most of the protons involved are bound and exchange readily with the bulk phases only when the thylakoids are swollen. It is concluded that, in shrunken thylakoids, the water-oxidizing enzymes are buried in special H^+-sequestering domains which probably are formed by cavities in the inner surface of the thylakoid membrane. An observed cooperative action of alkaline pH and divalent cations during Cl^--extraction from Photosystem II is interpreted as revealing an involvement of both a negatively charged surface region and positively charged groups in maintaining the functional integrity of the site of water oxidization.

Introduction

Photosynthetic water oxidation occurs at, or close to, the inside surface of the thylakoid membrane. This concept was invoked to explain the observation that an addition of protonophoretic or other uncoupling agents accelerated the inactivation of the water-oxidizing system in alkaline media [1,2]. It was assumed that the equilibration of protons between the external medium and the PS II site is rather sluggish unless uncouplers are added.

On the basis of experimental results obtained during the past several years, Dilley and co-workers [3,4] have suggested that the oxygen-evolving enzyme system resides in a special intramembranous domain towards the inside surface of the thylakoid membrane, and is separated from the lumen space by an H^+-impermeable barrier. Such a concept could explain the uncoupler-accelerated inactivations of the water-oxidizing system mentioned above, and those we have investigated recently [5,6]. Rather convincing support came from our analysis of the conditions required to lower the amount of functional Cl^- in the oxygen-evolving enzyme complex [6]. Among other things we were able to control the apparent pH at the water-

Abbreviations: Chl, chlorophyll; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

oxidizing site – as indicated by its ability to retain Cl^- – when we treated thylakoids with uncouplers at a given pH, and removed them prior to assays at a different pH [6]. Furthermore, as in the experiments of Dilley's group [4], electron transport through PS II appeared to be more effective in acidifying the PS II site than electron transport through PS I [7].

The combined data imply an existence of a metastable pH gradient in thylakoids after their transfer to a medium of lower acidity. Direct evidence for the persistence of such a pH differential between the thylakoid interior and the external bulk solution has been provided by Girault and Galmiche [8] through measurements of the distributions of added amines, and more recently by Dilley et al. [3] who showed that uncouplers cause the release of considerable amounts of protons from thylakoids which has been transferred to a more alkaline medium. We have confirmed this observation and demonstrated [5] that the amount of protons liberated from thylakoids initially stored in a medium of about neutral pH (6.5–7.0) was (1) only little affected by the type of buffer used in the storage medium, (2) not abolished when the assay medium contained high concentrations of Mg^{2+}, and (3) particularly pronounced in media of low osmolarity. We concluded that the uncoupler-released protons originated mainly from intrathylakoidal pools of bound protons which had not equilibrated with the external bulk solution. The size of these pools apparently varied with the conformational changes of the membranes that were caused by osmotically induced thylakoid swelling.

Because of its enormous magnitude (greater than 1 H^+/10 Chl), the uncoupler-induced proton release cannot be interpreted readily as an H^+ retention by the special PS II domains. We must assume that the deprotonation processes involve considerable portions of the thylakoids, and that the PS II domains are at best only a fraction of the large number of H^+-sequestering regions [5].

In a search for more information about the nature of the H^+ pools in thylakoids, and the interaction of those pools with PS II, we have now attempted to correlated the uncoupler-induced H^+ release and the destabilization of the oxygen-evolving systems in media of a pH larger than 7. We show that most of the protons originate from membrane-bound pools which equilibrate readily with the bulk phase of the thylakoid lumen only in swollen thylakoids. Furthermore, we corroborate the results of others that a negatively charged surface barrier exists between the water-oxidizing complex and the aqueous lumen space, and suggest that towards the inside from this surface region a certain degree of protonation is necessary for the maintenance of functional integrity in PS II.

Materials and Methods

Thylakoid preparations were obtained from leaves of vermiculite-grown pea plants (*Pisum sativum*, var. Progress 9). In general, the leaves were homogenized for 10 s at 3–5°C with a Waring blender in a medium containing 400 mM sucrose or sorbitol as osmoticum, 20–50 mM Na^+-Tricine (pH 7.6–8.2) or Na^+-Hepes (pH 6.8–7.6) buffer, plus 5 mM NaCl and 5 mM $MgCl_2$. The grinding medium contained also 3 mM sodium ascorbate when the thylakoids were to be used for PS II studies. $MgCl_2$ was omitted for analyses of Mg^{2+} effects, and only 1 mM NaCl was included when Cl^- deficiency was intended. After homogenization, the slurry was squeezed through miracloth, and the filtrate centrifuged for 10 min in a clinical centrifuge. The pellet was then washed twice in a wash medium which usually differed from the isolation medium. When low buffering capacity was desired as in the H^+-release studies, the two washing media and the final suspension medium contained only 6 mM Na^+-Hepes (pH 7.0), and sorbitol was omitted to obtain osmotically swollen thylakoids. Otherwise, the buffer concentration in the wash media was 20 mM. The preparations were stored in the dark on ice for ususally more than 1 h before use.

Oxygen evolution was measured with a Yellow Springs Clark-type oxygen sensor in a setup described previously [9]. In order to induce Cl^- deficiency, 40 μl of the 1 mM NaCl containing thylakoid suspension were injected into 4 ml Cl^--free assay medium. Care was taken to keep ambient light at very low levels.

The H^+ release was determined as pH change with a model 1019 Beckman Research pH meter.

For such measurement, usually 0.2 ml thylakoid suspension (150–300 μg Chl) were injected in very dim light into 4.8 ml stirred assay medium which contained 3 mM $MgCl_2$, 3 mM KCl and some NaOH to attain the desired assay pH, and the pH changes before and after uncoupler injections were recorded [5].

The measured ΔpH was converted to ΔH^+ using the measured value of ΔpH upon addition of known amounts of HCl. Within the limits of each recorded pH change, a constant $\Delta pH/H^+$ ratio could be used without introducing significant errors. When ΔpH measurements were accompanied by simultaneous recordings of the fluorescence emission from added pH probes, 2.4 ml assay medium were placed into a spectrophotometer cuvette and all additions were scaled down accordingly. The ability of thylakoids to retain protons, i.e., presumably the 'leakiness' of the thylakoid membranes, varied from preparation to preparation, especially when plants of different age, or from different species, were used. There was generally a loss of H^+-retaining capacity during storage of a given preparation, but the reproducibility was usually within 10% during the course of measurements over a 2–3 h period.

The modified setup for fluorescence measurements has been described [10]. Amplification of the signal was accomplished with a Keithley No, 427 current amplifier. Excitation light was obtained from a xenon arc lamp and filtered through 7.37 and 7.51 Corning glass filters. To avoid this light causing any electron transport-driven acidification of the thylakoid lumen when measurements of the internal H^+ pool of thylakoids in the dark were desired, 8 μM DCMU was included in the assay medium. The fluorescence from 9-aminoacridine was isolated at 488.5 nm, and that of atebrin at 508 nm, with Oriel narrow-band interference filters.

Chlorophyll were determined according to the method of McKinney [11]. All chemicals used were obtained commercially.

Results

The nature of the intrathylakoidal H^+ pool

In an earlier article [5], we have shown that the osmolarity of the external bulk phase influences the amount of protons which uncouplers will release from thylakoids after their transfer to a more akaline medium. Fig. 1 shows traces obtained at two different temperatures with thylakoids stored either in the presence or in the absence of 400 mM sorbitol, and assayed in media containing, or lacking, sorbitol. Independent measurements of light scattering indicated that volume changes of the thylakoids due to their transfer to a new medium were essentially completed after 2 min, the time of uncoupler addition in the experiment of Fig. 1. It was not unexpected, therefore, that the kinetics of the H^+ release were predominantly determined by

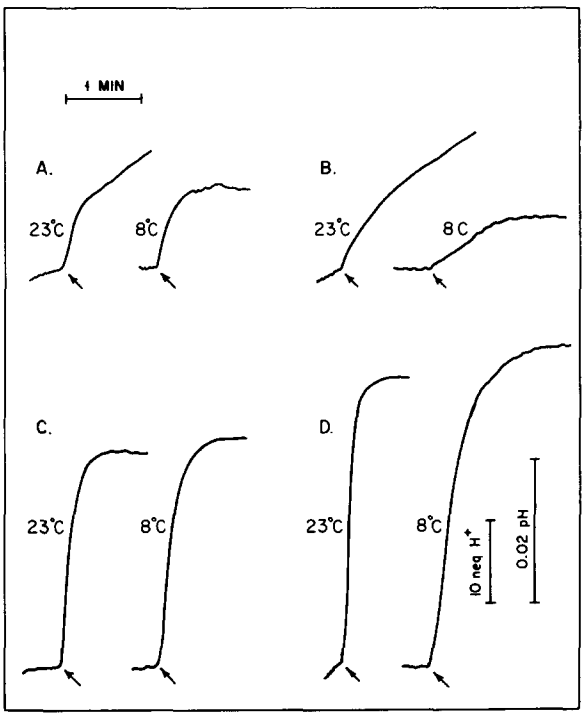

Fig. 1. Proton efflux from thylakoids in the dark. Assay as described in Materials and Methods at the indicated temperature. 2 min after injection of thylakoids into assay medium at the point indicated by the arrow, 1 μg/ml gramicidin D was added. pH of assay medium was between 8.32 and 8.44, and thylakoid concentrations were equivalent to 50 μg Chl/ml when stored in the presence of 400 mM sorbitol, or 45 μg Chl/ml when stored in the absence of sorbitol. (A) Thylakoids stored and assayed in the presence of 400 mM sorbitol; (B) storage in the absence, and assay in the presence of sorbitol; (C) storage in the presence, assay in absence of sorbitol; (D) storage and assay without sorbitol.

the composition of the assay medium.

For a discussion of the differences between the traces of Fig. 1 we will focus first on the recordings made at 23°C. Aside from differences in the magnitude of the pH change, the traces obtained in assay media containing sorbitol (A and B) indicate a continued acidification of the external medium even after completion of the uncoupler-induced H^+ liberation. The recording published by Dilley et al. [3] shows the same phenomenon. Since such acidification was not seen when the assay was performed in hypoosmotic media lacking sorbitol (traces C and D), we conclude that what may look like an artifactual 'pH-drift' at first glance, is in fact part of the H^+ equilibration between thylakoids and external bulk solution. Apparently, in osmotically shrunken thylakoids, some of the protons involved in the equilibration process do not exchange freely with those in the aqueous bulk phases. They may become exposed only during slow conformational changes that occur in response in the deprotonation of the surfaces of the thylakoid membranes. One test for this hypothesis was to measure the pH changes at a low temperature which would prevent or significantly retard any conformational rearrangements. We chose 8°C, a temperature well below 14°C at which an Arrhenius plot of the H^+ efflux from pea thylakoid membranes has been reported to show discontinuity [12]. The relevant traces of Fig. 1 show that, indeed, at such low temperature the uncoupler-insensitive acidification was essentially abolished. The gramicidin-induced H^+ release still occurred, but at a somewhat diminished rate.

We assume that, in contrast to osmotically shrunken thylakoids, in swollen thylakoids the protons capable of readily equilibrating with the surrounding aqueous phases comprise nearly the entire H^+ pools. One would expect, therefore, that in hypoosmotic media the sum of the continuous 'leakage' of protons prior to addition of uncouplers, and the uncoupler-released protons, is independent of the time of uncoupler addition. As shown in Fig. 2, this was the case. The displayed experiment includes one recording which was obtained with FCCP substituting for gramicidin as uncoupler to show the previously reported exchangeability of the uncouplers (see Ref. 5).

Experiments on the H^+ stoichiometry under

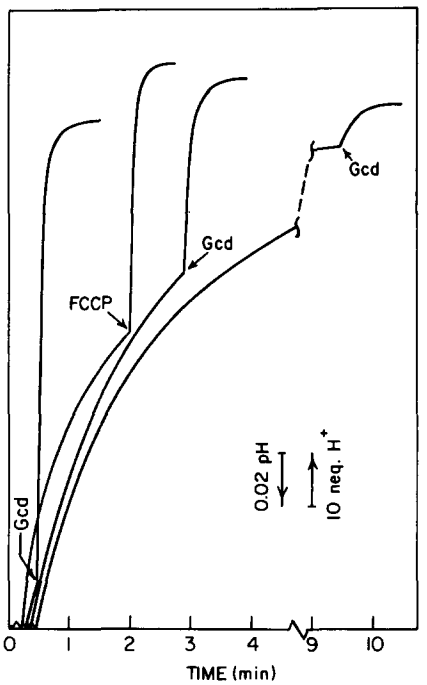

Fig. 2. Stoichiometry of total proton efflux from thylakoids in the dark. Thylakoids were stored and assayed in sorbitol-free media. pH of media during assay between 8.05 and 8.30; 40 μg Chl/ml; room temperature. At indicated times, 1 μg/ml gramicidin D (Gcd) or 5 μM FCCP was injected. The discontinuity at the beginning of the traces reflects the time required for the stabilization of the trace after thylakoid injection.

conditions which were different with respect to the osmolarities of the storage and assay media suggested that thylakoids contained larger metastable H^+ pools when they were swollen (not shown). Hence, shrinking and swelling would require and adjustment of the H^+ pools by exchange with protons of the surrounding bulk phases. This may explain why the acidifying drift was particularly spectacular when swollen thylakoids was injected into a medium of high osmolarity (Fig. 1B), and was essentially absent when, conversely, shrunken preparations were transferred to hypoosmotic conditions (see Fig. 1C). It is quite likely that such deprotonation and protonation processes also involve the external thylakoid surfaces.

Fig. 3 and Table I present data as proof for our claim [5] that the H^+ pools are only slightly sensitive to high concentrations of Mg^{2+} or K^+, and that their size depends on the pH of the storage

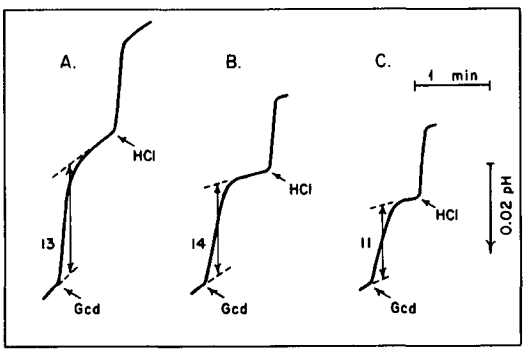

Fig. 3. Lack of effect of high salt concentrations on the gramicidin-induced proton efflux from thylakoids in the dark. Determination at room temperature at pH 8.3 as described for Fig. 1 with (A) no salt addition to the assay medium; (B) presence of 50 mM $MgCl_2$, and (C) presence of 120 mM KCl. Thylakoids were stored and assayed in the presence of sucrose; 45 μg Chl/ml per assay. At the first arrow, addition of 1 μg/ml gramicidin D (Gcd), at second arrow of 2 nmol/ml HCl. Numbers indicate nequiv. H^+/mg Chl.

medium regardless of its osmolarity. These results support the contention that the protons measured in our experiments are not adventitiously bound as a consequence of the membrane surface charge, and that, during storage, an equilibration occurs between the protons of the thylakoids and the suspension medium.

TABLE I

UNCOUPLER-INDUCED H^+ RELEASE FROM THYLAKOIDS IN THE DARK AS A FUNCTION OF THE STORAGE pH

Experimental conditions as described in Materials and Methods. Time of addition of gramicidin D (1 μg/ml), 2 min after injection of thylakoids. $T \approx 25°C$; 66 μg Chl/ml in the − sorbitol experiments, and 40 μg Chl/ml in the + sorbitol experiments.

Storage		Assay		
pH	± sorbitol	± sorbitol	nequiv. H^+/mg Chl	
			pH 8.23 ± 0.1	pH 9.07 ± 0.1
6.8	−	−	184	289
8.0	−	−	38	174
6.5	+	+	28	62
8.0	+	+	14	33

The data presented thus far imply that thylakoids, even after transfer into a medium of different pH, retain for a considerable time an internal pH which reflects that of their storage medium. It was of interest, therefore, to verify that such a pH gradient did indeed exist under our conditions. For this reason, we analyzed the response of fluorescent amines in our system, and compared it to the amount of uncoupler-released protons. Figs. 4 and 5 display traces drawn from simultaneous recordings of probe fluorescence and pH changes. One experimental difficulty turned out to be the H^+-releasing effect of the fluorescent amines, which increased with the time that had elapsed after thylakoid preparation. Moreover, as mentioned in our previous report [5], some uncoupler-like effect of DCMU also had to be considered, especially at the relatively high pH of these measurements. Yet, the result was clear: An uncoupler-sensitive quenching of the probe fluorescence correlated well with the amount of uncou-

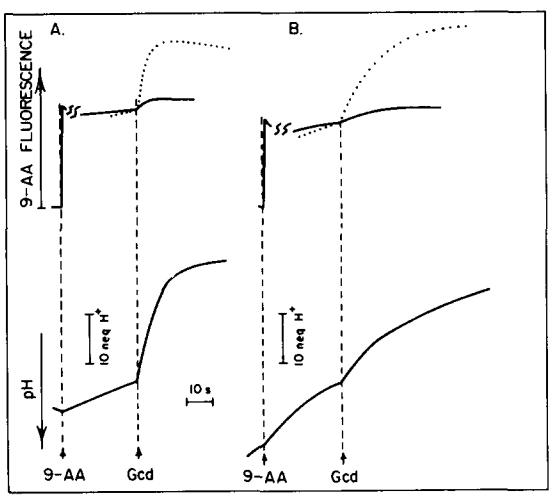

Fig. 4. Simultaneous determinations of gramicidin-induced proton efflux from thylakoids in the dark, and change of 9-aminoacridine fluorescence. In Expt. A, thylakoids were stored and assayed in sorbitol-free media (37 μg Chl/ml), in Expt. B storage and assay were in the presence of 400 mM sorbitol (38 μg Chl/ml); assay at room temperature at pH ≈ 9.2. At arrow marked 9-AA, 1.2 μM 9-aminoacridine was injected, and at arrow marked Gcd, 1 μg/ml gramicidin D was added. All traces were redrawn to the same time scale from original recordings. The dotted lines are 10-fold amplified fluorescence signals from different experiments to show the kinetics of the changes.

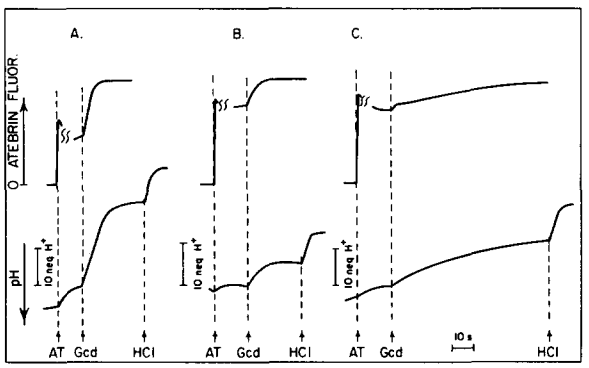

Fig. 5. Simultaneous determinations of gramicidin-induced proton effux from thylakoids in the dark, and change of atebrin fluorescence. In Expts. A and B, thylakoids were stored and assayed (35 μg Chl/ml) in the absence of sorbitol with gramicidin added at different times after transfer. Expt. C recorded with thylakoids stored and assayed in the presence of 400 mM sorbitol. At arrow marked AT, 1.5 μM atebrin added; at arrow Gcd, 1 μg/ml gramicidin D added, at arrow HCl 4 nmol/ml HCl added. pH during assay between 9.05 and 9.25; $T = 25°C$. Traces redrawn to identical time scale from original recordings.

pler-releasable H^+. A most surprising additional finding was that the slow H^+ release in sorbitol-containing media was paralleled by a similarly slow recovery of the probe fluorescence. This observation was incompatible with the concept that the amines report only the ΔpH between the bulk aqueous phases because the gradient of free H^+ should collapse rapidly upon uncoupler addition.

The slow responses of atebrin and 9-aminoacridine in media of high osmolarity differed in that the kinetics of the former were biphasic and quite slow, while the fluorescence of 9-aminoacridine changed with a seemingly monophasic time course. The biphasic nature of the fluorescence increase measured with atebrin after gramicidin addition probably indicated a rapid initial equilibration of the pH between the bulk phases, and a more sluggish release of bound atebrin. The more long-lasting change of the atebrin fluorescence may have reflected the fact that, by virtue of its higher pK_a, this probe will sense an internal pH close to the assay pH of about 9.2, while 9-aminoacridine would be much less able to do so. Another, as yet unexplained peculiarity, was a slow decline of the 9-aminoacridine fluorescence after its maximal intensity had been reached following gramidicin addition to sorbitol-containing suspensions of thylakoids. No such decline was seen in sorbitol-free media. The slow fluorescence quenching was not prevented by additions of more Mg^{2+} even though it caused an increase in the overall fluorescence emission (not shown). Hence, it is unlikely that 9-aminoacridine became attracted by negatively charged sites which had become exposed in the wake of the uncoupler-induced proton equilibration. Usually, such interactions between 9-aminoacridine and negatively charged membrane surfaces are competitively abolished by Mg^{2+} [13,14].

From the extent of the uncoupler-reversed fluorescence quenching, and with the appropriate equations of Schuldiner et al. [15], a pH gradient of approx. 2 was calculated using $V_e/V_i \approx 10^3$. This agreed well with our expectations, since the thylakoids were poised during storage at pH ≈ 6.8, and assayed at about pH 9.

pH-dependent inactivations of PS II

It is now well established that PS II inactivating procedures, when carried out at pH > 7, are accelerated in the presence of uncouplers [1–7]. We agree with Dilley et al. [3] that a relation exists between the metastable H^+ pools in thylakoid preparations, and uncoupler-stimulated interferences with PS II activity at elevated pH. To substantiate further this correlation, we have investigated the effect of osmotic conditions on the inactivation of PS II at high pH. It can be seen from Fig. 6 that it was accelerated considerably in the absence of the osmoticum sorbitol, and that the kinetics of the process were quite different, being exponential in the absence of sorbitol, and linear in its presence. This parallels the gradual course of the H^+ equilibration seen after gramicidin addition to sorbitol-containing media as well as the fast and complete H^+ efflux induced by uncouplers in hypotonic suspensions (see Fig. 1). Such a correlation between PS II inactivation and the properties of the proton pools was also evident when the alkaline treatment was performed at a lower temperature. In a sorbitol-containing medium, PS II activity was almost completely retained during a 4 min incubation at pH 9.6, while under

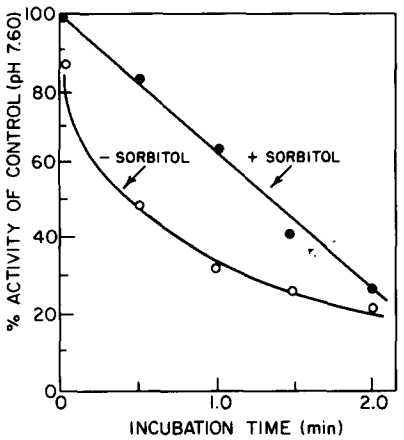

Fig. 6. Inactivation of PS II activity at alkaline pH. Thylakoids (25 μg Chl/ml) in 20 mM Na^+-Hepes, pH 7.6, 3 mM $MgSO_4$, 3 mM NaCl plus 0.8 μg/ml gramicidin D at 23°C were brought to pH 9.6 with NaOH and incubated for various periods. The pH was then returned to 7.6 with HCl, and the Hill activity measured with 1.2 mM potassium ferricyanide in saturating white light. 400 mM sorbitol present where indicated.

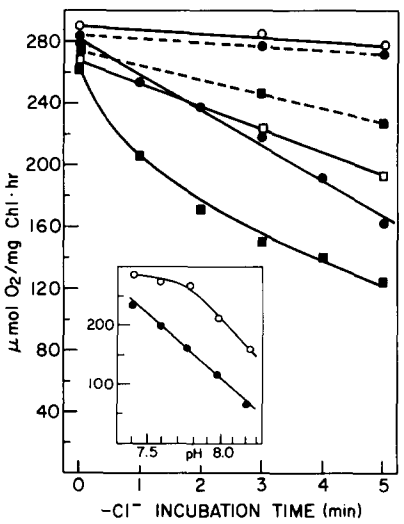

Fig. 7. Loss of PS II indicated due to Cl^- deficiency as a function of osmolarity and Mg^{2+}. Thylakoids prepared and stored at pH 7.2 as described in Materials and Methods in the absence or presence of 400 mM sorbitol. Incubation medium as for Fig. 6 except for pH 7.65. NaCl was omitted in all assays except those depicted as dashed lines. (■ and □) Sorbitol absent, (□ and ○) $MgSO_4$ absent. Dark incubation for various times prior to measurement of Hill activity with 1.2 mM ferricyanide in saturating white light. Inset: pH dependence of loss of PS II activity due to Cl^- depletion in the presence and absence of Mg^{2+} using as buffer a mixture of 10 mM Na^+-Tricine and 10 mM Na^+-Hepes.

the same conditions an activity loss of approx. 30% occurred in a sorbitol-free suspension. This finding would be expected because, as shown above, lowered temperatures considerably retard the liberation of the slowly equilibrating H^+ pools which are prevalent in high-osmolarity media.

While treatment of thylakoids at high pH causes an irreversible inactivation of the water-oxidizing system, presumably as a consequence of manganese loss [13], the removal of functional Cl^- from PS II is reversible [16]. Yet, also Cl^- extraction is accomplished most easily under conditions which allow alkalinization of the interior of the thylakoids. In our previous studies, we have achieved Cl^- deficiency by incubating thylakoids in slightly alkaline Cl^--free media which contained an uncoupler and were kept in darkness [6]. In order to establish whether the metastable H^+ pools also affect the kinetics of Cl^- release, we measured the reversible activity loss of thylakoids in Cl^--free media containing or lacking sorbitol. Again, the decline of PS II acitivity was much faster in the absence than in the presence of 400 mM sorbitol (Fig. 7). One very unexpected discovery in the course of our investigation was that an omission of Mg^{2+} from the Cl^--free incubation medium protected the thylakoids against Cl^- loss, especially when the osmolarity of the medium was high. The effect of Mg^{2+} is clearly evident when the appropriate traces in Fig. 7 are compared. The inset in Fig. 7 shows that the action of Mg^{2+} was mainly to lower the threshold of the pH needed for creating Cl^- deficiency.

A further test revealing the probably general ability of divalent cations to facilitate Cl^- release from PS II was based on our previous observation that, in the presence of low or moderate amounts of uncouplers, light-driven electron transport prevents Cl^- loss. A short interruption of the illumination by darkness was all that was needed to disperse the protons deposited by electron transport and create conditions necessary for Cl^- efflux from its functional site [6]. With Mg^{2+} as the divalent cation, we demonstrate with the data shown in Table II that the addition of a chelator before, but not after, the dark period prevented PS

TABLE II

PREVENTION OF Cl^- RELEASE FROM PS II BY EDTA ADDITION TO Mg^{2+}-CONTAINING MEDIA

Thylakoids isolated and assayed (25 μg Chl/ml) at pH 7.8 (see Materials and Methods) with 1.2 mM ferricyanide as electron acceptor in saturating white light. The complete reaction mixture contained 400 mM sorbitol, 20 mM Na^+-Hepes, 3 mM $MgSO_4$, 3 mM NaCl. 0.8 μg/ml gramicidin D were added at the beginning of the light period which, after 90 s, was interrupted by 75 s darkness. 6 mM EDTA was added either before or at the end of the dark period.

NaCl	EDTA added	μmol O_2/mg Chl per h	
		Before dark	After dark
+	–	198	159
–	before dark	222	156
–	after dark	228	126

II inactivation due to Cl^- depletion. Similar results were obtained when Mg^{2+} was replaced by Ca^{2+} (not shown).

Discussion

This work confirms earlier data [1–7,17] which had indicated that the equilibration of protons in the dark between thylakoids and the surrounding medium can be remarkably slow. We followed the equilibration process by measuring the acidification of a thylakoid suspension after thylakoids had been transferred to an assay medium slightly more alkaline than their storage medium. Surprisingly, uncoupler addition accelerated the entire equilibration process only in osmotically swollen thylakoids, while in shrunken thylakoids a considerable portion of protons became only gradually involved regardless of the presence of protonophoretically active agents. On the basis of our measurements with fluorescent probes, and of our H^+-release experiments at 8°C, we attribute the difference between shrunken and swollen thylakoids mainly to the relative abundance of those H^+-binding groups which are in direct equilibrium with the lumen bulk phase. This is shown schematically in Fig. 8. The nature of the H^+-binding groups cannot be identified at this time, but in the pH range of our experiments only very basic groups like amine derivatives are conceivable as sufficiently rich H^+ sources. This agrees with the observed unmasking of amino groups for acylation when thylakoids were transferrred in the dark to uncoupler-containing hypoosmotic media pH 8.5 [3,4].

The steady, uncoupler-independent H^+ release from shrunken thylakoids can be explained if one assumes that alterations of the membranes' surface charge in the wake of the pH equilibration process cause conformational changes which expose previously buried protonated groups, perhaps with a concomitant change of their pK. In fact, differences in the physical properties of thylakoid membranes under different osmotic conditions were suggested by our measurements at 8°C. It is known that the conductivity of lipid bilayers in the presence of gramicidin is temperature insensitive as long as the bilayer is fluid, but becomes temperature sensitive below the transition temperature [18]. From the traces of Fig. 1 we can see that the effect of temperature on the gramicidin-dependent H^+ efflux was smallest for shrunken thylakoids suspended in a cold hypoosmotic medium, and largest for swollen thylakoids injected into a cold solution of 400 mM sorbitol. Apparently, the former assumed some 'super-rigid' character, while the latter behaved like a normal membrane even at 8°C.

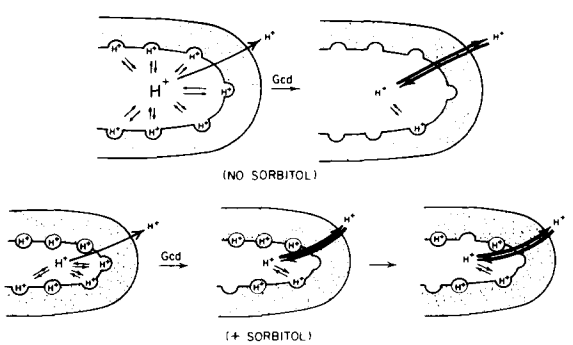

Fig. 8. Schematic representation of the difference between swollen and shrunken thylakoids in respect to their metastable protons. Encircled H^+ do not readily equilibrate with the bulk phase (see text). Gcd, gramicidin D.

Using fluorescent amines, we were able to visualize directly the metastable pH gradient in our thylakoid preprations. Yet, the slow or even biphasic recovery of the probe response seen when protons were allowed to equilibrate in media of high osmolarity shed doubt on the contention that the probes reported solely the pH gradient between the internal and external aqueous bulk phases. Such ambiguity of the probe response has been suggested by several investigators [19]. Preliminary analyses using an approach developed by Haraux and De Kouchovsky [14] have suggested that the binding ratio $[H^+_{total}]/[H^+_{free}]$ for the thylakoids was constant. While this result agreed with that of the just-mentioned authors who measured light-dependent H^+ uptake, it cannot be reconciled with our previously reported [5] exponential dependence of the amount of releasable protons on the external pH. In fact, as Haraux and De Kouchovsky have already pointed out, a constant binding ratio is, in itself, a rather unrealistic phenomenon. We suggest, therefore, that much of the observed response reflected interactions at the interface between the bulk phases and membrane surfaces, and involved both protons and fluorescent probes.

The correlation between the metastable H^+ pool(s) in the thylakoids and PS II function was striking. We demonstrated that pH-dependent inactivations of the water oxidizing enzyme were particularly rapid in hypoosmotic media, and found a complete stabilization of PS II under conditions of high osmolarity at 8°C. These results would be predicted if the protons needed for PS II stabilization are in a buried position whenever the thylakoids are not swollen. We have shown that the equilibration of such protons can be retarded effectively at lowered temperatures.

Parallels were also found between H^+ equilibration and the release of functional Cl^- from PS II. In this case we made the surprising additional observation that Ca^{2+} or Mg^{2+} was required when Cl^- deficiency was to be induced at pH $\leq$ 7.8. A need for Mg^{2+} became less apparent in media of higher pH or hypotonic media, suggesting a cooperative function of divalent cations when $[OH^-]$ was suboptimal and the protonated Cl^- site was obstructed.

It is known that the water-oxidizing sites are located at, or close to, a negatively charged region of the inner surfaces of thylakoid membrane [20,21]. The sensitivity of these sites, and their associated Cl^-, to slightly alkaline conditions suggests, furthermore, a presence of groups with rather high pK_a, e.g., amines. As a preliminary explanation of our data we propose that the water-oxidizing enzyme is located is depressions of the thylakoid membrane's inner surface. Adopting recently published knowledge about the organization of the manganese complex [22], the sketch of Fig. 9 is a graphic representation of our concept. In it, the water-oxidizing cavity is thought to be lined with amino groups of which most are usually protonated and which may serve as H^+-conductors during water oxidation. The mouth of the cavity, in contrast, has a net negative charge. Cl^- may serve as counterion to the net positive charge in the cavity. Hence, a deprotonation of the amino groups is a prerequisite for Cl^- release. The unprotonated amino groups are then available as ligands for the manganese in the water-oxidizing enzyme where they may break oxygen linkages in the same way as added amines might be assumed to interfere with the function of the manganese-containing enzyme [23]. Divalent cations can be expected to shield the negatively charged region that separates the cavity from the bulk medium in the thylakoid lumen. This increase in net positive charge might lower the amines' pK_a and thus allow even at relatively low $[OH^-]$ a deprotonation sufficient for Cl^- release. At the same time, the

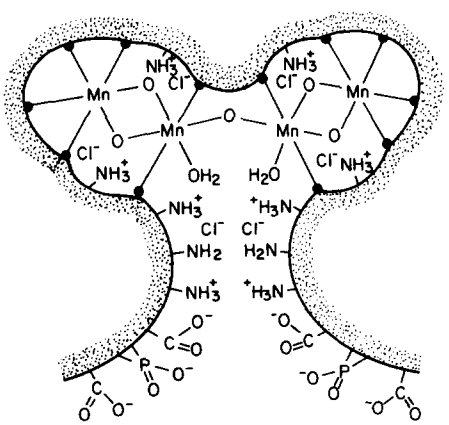

Fig. 9. Schematic representation of the possible organization of the cavity harboring the water-oxidizing enzyme (see text).

lowered negative surface charge towards the lumen will facilitate Cl^- extrusion. The deleterious effect of more alkaline conditions may well involve similar events with the added complications that manganese itself becomes released [16].

We believe that our data provide support for the concept of a special H^+-sequestering domain around PS II as postulated by Dilley and coworkers [3,4], and adopted by us [6]. However, on the basis of our new observations we consider these domains to be neither truly intermembranous, nor the sole contributors to the metastable H^+ pools measurable in thylakoids. The physiological significance of a buried oxygen-evolving complex, and of the osmotic control of its properties, remains to be elucidated.

Note added in proof (Received April 7th, 1983)

According to recent results (Pfister, V.R. and Homann, P.H., unpublished data), our contention that protonated buffer molecules trapped in the thylakoid lumen did not significantly contribute to the measured H^+ retention is correct only for thylakoids in their shrunken state, i.e., in the presence of sucrose or sorbitol.

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