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STUDIES ON OXYGEN EVOLUTION OF INSIDE-OUT THYLAKOID VESICLES FROM MANGROVES: CHLORIDE REQUIREMENT, pH DEPENDENCE AND POLYPEPTIDE COMPOSITION

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Inside-out thylakoid vesicles from the halophyte *Avicennia marina* were isolated by the aqueous polymer phase partition method. Oxygen-evolution activity measured with ferricyanide and phenyl-*p*-benzoquinone was absolutely dependent on added chloride, since the vesicles were almost completely depleted of the 23 and 16 kDa polypeptides of the O_2-evolving complex. Addition of the spinach 23 kDa protein to the mangrove inside-out vesicles lowered their chloride requirement for O_2 evolution at least 50-fold. In the absence of added chloride, the mangrove vesicles were very sensitive to inhibition by light, which could be prevented by high chloride or low chloride plus added purified spinach 23 kDa protein. The preparations were also inactivated by neutral or alkaline pH (greater than 7.2) in the absence of high chloride concentrations. This inactivation was not significantly influenced by addition of spinach 23 kDa protein. Chloride binding and alkaline inhibition may therefore be closely related, either directly via the manganese centers or, more likely, via pK_a changes in as yet unidentified proteins.

Introduction

Since the development of techniques for preparation of O_2-evolving inside-out thylakoid vesicles [1–3] and Photosystem II (PS II) particles [4–7], considerable advances have been made in our understanding of the structure and function of the O_2-evolving complex. Its localization on the inner thylakoid surface [8], the requirement for manganese [9] and chloride [10,11], and its pH sensitivity [12–15] have been known for quite some time. Only recently, however, has it been possible to identify three polypeptides of approx. 33, 23 and 16 kDa which are functionally associated with the O_2-evolving complex [16]. The roles of the 33 and 16 kDa polypeptides have not so far been established, although the 33 kDa protein may be directly or indirectly associated with manganese [17–19], while the 16 kDa protein may be involved in calcium binding to the O_2-evolving complex [20–22]. By reconstituting the purified spinach 23 kDa protein with inside-out vesicles from mangrove thylakoids, this protein was recently shown to modify the chloride requirement of O_2 evolution [23].

Thylakoids as well as inside-out thylakoid vesicles from salt-tolerant species such as man-

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Abbreviations: PS, photosystem; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; AMPD, 2-amino-2-methyl-1,3-propanediol; PBQ, phenyl-*p*-benzoquinone; Chl, chlorophyll; Mes, 4-morpholineethanesulphonic acid.

groves have structural and functional properties which are quite different from those of spinach or other non-salt tolerant plants [11,15,23,24]. These properties have, on the one hand, been exploited in elucidating aspects of the mechanism of O_2 evolution, but may, on the other hand, also be a manifestation of an adaptation to salinity at the molecular (biochemical) level. Keeping in mind both those mechanistic and physiological aspects, we present results in this paper concerning the chloride requirement of O_2 evolution in inside-out thylakoid vesicles from mangroves (*A. marina*), its pH dependence and polypeptide requirements. Additionally, the effects of preillumination on O_2-evolution activity of thylakoids are reported.

Material and Methods

Thylakoids from 50–100 g mangrove leaves (*Avicennia marina*, collected from a mangrove swamp in Cullendulla Creek, Bateman's Bay, N.S.W., Australia) were prepared by grinding 2–5 mm^2 sections in 150–250 ml of ice-cold 50 mM Hepes-AMPD (pH 7.3)/500 mM sorbitol/5 mM dithiothreitol, in a Janke & Kunkel Ultra-Turrax for 30 s at top speed. The homogenate was filtered through four layers of Miracloth and centrifuged at 200 × *g* for 5 min. The supernatant suspension was recentrifuged at 20000 × *g* for 10 min and the pellet washed once (10 min, 13000 × *g*) in buffer comprising 10 mM sodium-phosphate (pH 7.4)/5 mM NaCl/5 mM $MgCl_2$/100 mM sucrose. The final pellet of mangrove thylakoids was resuspended in the above washing buffer.

Preparation of inside-out mangrove thylakoid vesicles was carried out essentially as described for spinach [3] by aqueous polymer two-phase partition [25], using a phase system composed of 5.7% (w/w) Dextran T-500/5.7% (w/w) poly(ethylene glycol) 4000 (Carbowax 3350)/10 mM sodium-phosphate buffer (pH 7.4)/5 mM NaCl/20 mM sucrose. These procedures yielded fractions T2 (right-side out) and B2 (inside-out), respectively, which were diluted with 5 mM sodium-phosphate buffer (pH 7.4), 500 mM sucrose, 2.5 mM NaCl, and the vesicles pelleted by centrifugation at 100000 × *g* for 60 min.

O_2-evolution activity of the vesicles was measured as described previously [23]. Variables such as pH, chloride concentration or other additions are given in the figure legends.

Light-induced proton translocation was measured in a temperature-regulated O_2 electrode chamber at 25°C, illuminated with red-filtered light from a slide projector, using a custom-made pH meter and a combined Radiometer GK 2321C electrode connected to a chart recorder. The pH changes were calibrated by adding known amounts of 1 mM NaOH. Assays were carried out in unbuffered 100 mM NaCl, containing in 1 ml final volume 0.1 mM PBQ and 0.2 mg Chl.

Reconstitution experiments with purified spinach proteins, gel electrophoresis and Western blotting were performed as described before [23].

Results

Aqueous polymer two-phase partition following Yeda press fragmentation of mangrove thylakoids yielded two distinct subfractions with different affinity for the two phases. The two thylakoid subpopulations showed opposite direction of their light-dependent proton translocation (Fig. 1). The thylakoid membrane vesicles partitioning to the lower phase (B2) showed light-induced proton extrusion typical of inside-out vesicles, while the material of the upper phase showed proton uptake typical of thylakoids with normal sidedness. Thus, the phase system can separate inside-out and right-side-out vesicles from mangrove thylakoids in the same way as originally described for spinach [1–3].

In inside-out thylakoid vesicles, the water-split-

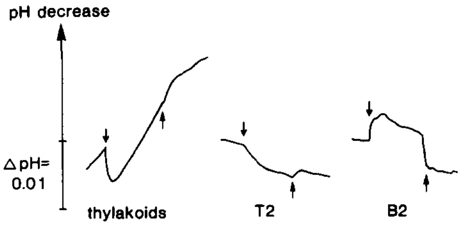

Fig. 1. Light-induced proton movement mediated by 0.1 mM PBQ in thylakoids, right-side-out (T2) and inside-out (B2) vesicles from mangroves. Initial pH was 6.6. Proton translocation (nmol H$^+$/mg Chl): thylakoids, −80; T2, −3, 4; B2, +2, 1.

ting site is exposed to the surrounding medium and not shielded behind the membrane barrier as is the case in normal thylakoids. Thus, in the everted vesicles, water oxidation is directly accessible for various treatments or addition of effectors. Fig. 2 compares the rates of oxygen evolution at increasing NaCl concentrations in the two vesicle populations. Oxygen evolution of the right-side-out vesicles responds to salt in essentially the same way as has previously been demonstrated for thylakoids [11,15,24], in showing very low rates below 50 mM NaCl, while optimal activities required about 250 mM. Strikingly, the inside-out vesicles showed the same general salt dependence and tolerance. In fact, the salt response for the inside-out vesicles was even more pronounced than for the right-sided vesicles. At very low salt concentrations (up to 30 mM), the inside-out vesicles showed virtually no activity, while the right-side-out had some. This can probably be explained by residual chloride remaining in the right-side-out vesicles. The somewhat higher activity at optimal NaCl concentrations in the everted vesicles may be explained by their Photosystem II enrichment compared to the right-side ones.

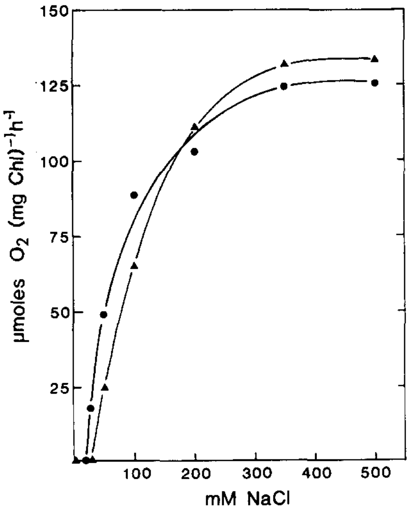

Fig. 2. Dependence of O_2-evolution activity of right-side-out vesicles (●———●) or inside-out vesicles (▲———▲) on NaCl concentration in the assay medium. 2 ml total volume of 25 mM Hepes-AMPD, (pH 7.8)/1 mM ferricyanide/0.2 mM PBQ/30 μg Chl.

The salt response curve for oxygen evolution in the mangrove inside-out vesicles was drastically changed upon addition of spinach 23 kDa protein which decreased the NaCl requirement for maximal oxygen evolution significantly [23]. In Fig. 3, O_2-evolution traces are shown which confirm and extend the earlier observations: in the absence of the protein, very little or no activity was observed in inside-out thylakoid vesicles at low (2.5 mM) chloride concentration and high activity was measured only at high chloride concentration (250 mM). When the 23 kDa protein was added and allowed to rebind to the exposed inner thylakoid surface, optimal rates were obtained already at 2.5 mM NaCl. The NaCl requirement for half-maximal activity without the 23 kDa protein was around 25 mM, while the value in the presence of the protein was below 0.5 mM. Thus, the 23 kDa protein lowers the chloride requirement for oxygen evolution in inside-out vesicles at least 50-fold. Fig. 3 also illustrates the chloride specificity, since equal amounts of SO_4^{2-} failed to give any increase in activity in the presence of added protein. Conversely, the effectiveness of the 23 kDa protein was shown by the lack of activity upon addition of a heated protein aliquot.

A comparative analysis of the 33, 23 and 16 kDa proteins in spinach and mangrove thylakoids by Western blotting immunoelectrophoresis using a mixture of the three monospecific antisera [23] is shown in Fig. 4. Although all three polypeptides

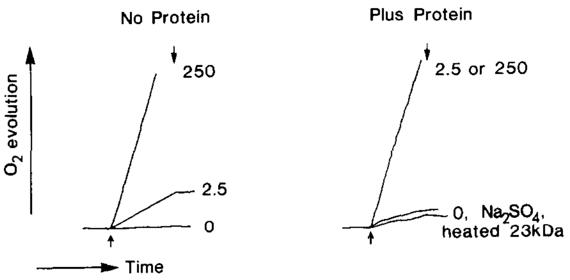

Fig. 3. O_2-evolution traces demonstrating the modulation of the chloride response in inside-out thylakoid vesicles from mangroves by addition of purified spinach 23 kDa or 23 plus 16 kDa protein. Numbers adjacent to traces indicate mM NaCl added. Assay conditions as in Fig. 2, except pH 6.8; 7.5 μg Chl/ml; added polypeptide fractions were 0.25 mg protein/mg Chl. ↑ light on, ↓ light off.

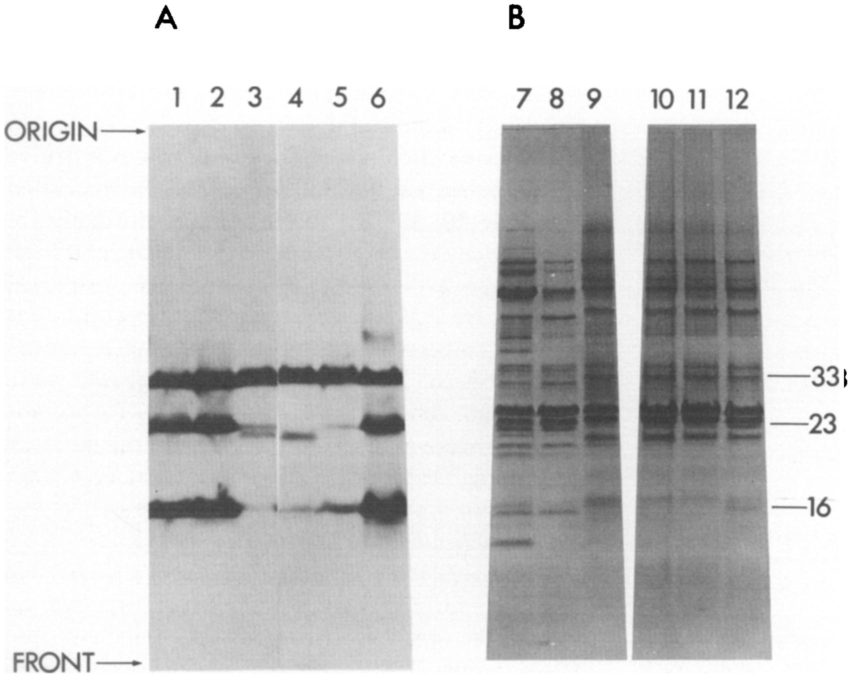

Fig. 4. Detection of the 33, 23 and 16 kDa polypeptides of Photosystem II by Western blot analysis and Coomassie staining. Lanes 1–6: immunological detection using combined antisera against the 33, 23 and 16 kDa proteins; lanes 7–12: stained polypeptide patterns. Lanes 1, 7, spinach thylakoids; lanes 2, 8, inverted spinach thylakoid vesicles; lanes 3, 9, mangrove thylakoids; lanes 4, 10, inverted mangrove thylakoid vesicles; lanes 5, 11, inverted mangrove thylakoid vesicles washed with 250 mM NaCl; lanes 6, 12, salt-washed inverted mangrove thylakoids reconstituted with the 23 and 16 kDa proteins from spinach.

were present at high levels in both thylakoids and inside-out vesicles (B7) from spinach (lanes 1 and 2), the mangrove thylakoids used here (lane 3) and the everted vesicles prepared from them (lane 4) contained much reduced quantities of the 23 and 16 kDa proteins. This shows that these proteins are released from mangrove thylakoids during isolation at low osmotic strength, since they are retained where isolation is carried out in the presence of 1 M sorbitol [23,26]. Reconstitution of the salt-washed, everted mangrove vesicles with the purified 23 and 16 kDa proteins from spinach resulted in stable binding of both proteins to the membrane (lanes 5 versus 6), concomitant with the drop in the Cl^- requirement for oxygen evolution. The immunological results were confirmed by Coomassie staining of the equivalent lanes (Fig. 4, lanes 7–12), which again demonstrated the almost complete absence of the 23 and 16 kDa bands from mangrove vesicles (lanes 9–11) and the rebinding of the spinach polypeptides under reconstitution conditions (lane 12).

Illumination of photosynthetic membranes in the absence of chloride has variously been described to be beneficial [27] or deleterious [29]. In mangrove thylakoids, illumination with saturating light (as used for O_2-evolution assays) in the absence of added chloride resulted in irreversible inactivation of the O_2-evolution activity (Fig. 5, see also Ref. 24). This inactivation was time and pH dependent, as shown in Fig. 5A and B, respectively. Inside-out mangrove thylakoid vesicles are equally as sensitive to preillumination in the absence of chloride (Table I). Even at pH 6.8, 2 min preillumination was sufficient to abolish O_2-evolution activity subsequently measured at 50 mM NaCl. The major portion of this inactivation was observed after preillumination at 2.5 mM NaCl. However, 50 mM NaCl protected against the inactivation by preillumination, demonstrating that the lack of chloride was responsible for the deactivation. Strikingly, the presence of the 23 kDa protein during preillumination at 2.5 mM NaCl also protected against inhibition. No effect was seen if the protein was added after the preillumination period. Spinach inside-out vesicles, which retain the 23 and 16 kDa proteins (Fig. 4) and chloride were not at all inhibited by preil-

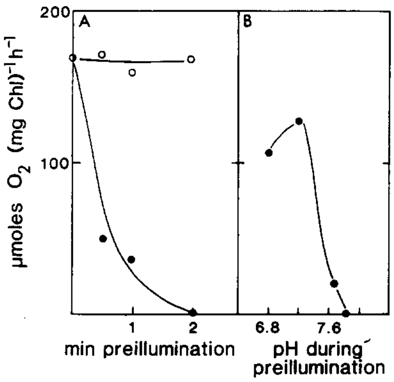

Fig. 5. (A) Inhibition of O_2-evolution activity in mangrove thylakoids as a function of preillumination time in the absence of electron acceptors and added chloride at pH 7.8 (●———●); controls illuminated in the absence of electron acceptors but with 500 mM NaCl at pH 7.8 (○———○). Assays in 25 mM Hepes-AMPD (pH 7.8)/500 mM NaCl/1 mM ferricyanide/0.1 mM PBQ/20 µg Chl. (B) pH dependence of inhibition by 1 min preillumination in the absence of added chloride and electron acceptors of O_2-evolution activity in mangrove thylakoids. Assays at same pH as preillumination, otherwise conditions as in Fig. 6A.

lumination in the absence of added chloride. In fact, 3.5 min preillumination at pH 6.3 in the absence of added chloride resulted in a marginally higher activity when subsequently assayed in 50 mM NaCl, as compared to preillumination at pH 6.3 in the presence of 50 mM NaCl (251 versus 236 µmol O_2/mg Chl per h, respectively). Only after inside-out spinach vesicles were depleted of the 23 and 16 kDa polypeptides by salt-washing did they become inhibited by preillumination in the absence of chloride. They were not as sensitive as the mangrove vesicles, which may be explained by some 10–30% of these proteins normally remaining on the membrane [28]. Partial inhibition of oxygen evolution in the mangrove inside-out vesicles by light under low chloride conditions could also be seen during the course of a normal assay (Fig. 6). Thus, although the initial rates were quite high, they dropped continuously to become nearly inactivated within a couple of minutes. Steady high rates were only obtained at high chloride concentrations or at low chloride concentrations when the 23 kDa protein was present.

A particularly intriguing feature of the chloride requirement of O_2 evolution is its pH dependence. From the early experiments by Gorham and Clendenning [29] to the more recent observations [10,11,15,24], the results consistently show an increase in chloride requirement for O_2-evolution function at pH above 7.2–7.4. Conversely, at pH below 6.5–6.8 there is no absolute chloride requirement in some systems (Ref. 15, but see also Ref. 10). In mangrove thylakoids, this chloride requirement at slightly acidic pH is variable and was previously suggested to be dependent on the amount of residual chloride remaining in individual preparations [15]. Inside-out thylakoid vesicles of mangroves were considered to be completely

TABLE I

PREILLUMINATION OF INSIDE-OUT MANGROVE THYLAKOID VESICLES IN THE ABSENCE OR PRESENCE OF CHLORIDE

Assay conditions as in Fig. 3. Rates in µmol O_2/mg Chl per h.

Preillumination condition	Assay condition	Rate
2 min, pH 6.8	50 mM NaCl	0
2 min, pH 6.8, 2.5 mM NaCl	50 mM NaCl	49
2 min, pH 6.8, 50 mM NaCl	50 mM NaCl	173
2 min, pH 6.8, 2.5 mM NaCl, 23 kDa	2.5 mM NaCl	173
2 min, pH 6.8, 2.5 mM NaCl	2.5 mM NaCl, 23 kDa	72

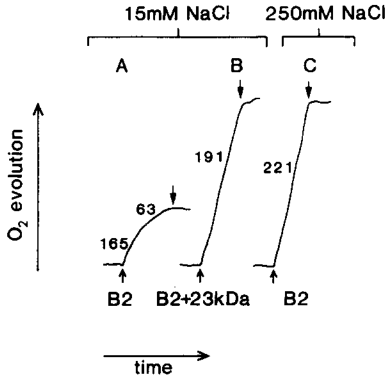

Fig. 6. Kinetics of O_2 evolution in mangrove inside-out thylakoid vesicles when assayed at low (15 mM) (A), high (250 mM) (C) or low (15 mM) chloride concentration plus 23 kDa (B). Assay conditions as in Fig. 3.

chloride-deficient, since no residual activity was measurable in the absence of added chloride even at pH 6.3 or 6.8. Low amounts of chloride, however, were effective in stimulating high activity at low pH (Fig. 7). O_2-evolution activity at low (2.5 mM) chloride concentrations declined rapidly with increasing pH and was completely abolished at pH 7.2 (Fig. 7). A normal pH optimum curve was measured in the presence of 250 mM added NaCl. The pH optimum under these conditions was at pH 7.0 (Fig. 7), the rates falling off symmetrically on both sides of the optimum. Addition of the purified spinach 23 kDa protein to the inside-out mangrove vesicles shifted the pH optimum considerably towards the acidic region and at 2.5 mM NaCl it was around pH 6.3 (Fig. 7). The binding of the 23 kDa protein to the membranes (see also Fig. 4) at low chloride concentration did not greatly alter the inhibition by pH above 7.0–7.2, as can be seen from Fig. 7 also. High chloride concentrations were much more effective in preventing this alkaline inhibition.

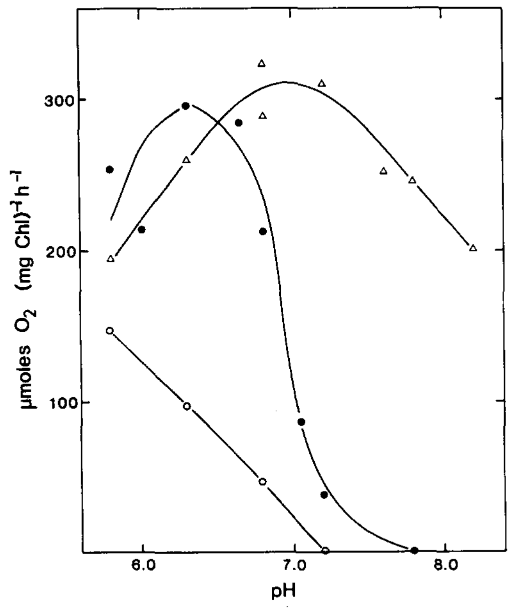

Fig. 7. pH dependence of O_2-evolution activity in inside-out thylakoid vesicles from mangroves when assayed in the presence of low (2.5 mM) (○———○) or high (250 mM (△———△) chloride concentration, or at low (2.5 mM) chloride concentration in the presence of the spinach 23 kDa protein (●———●). Assay conditions as in Fig. 2. pH 5.8–6.3, Mes; pH 6.8–8.2, Hepes.

Discussion

In inside-out thylakoid vesicles, the water-splitting site is exposed to the surrounding medium. It was therefore of interest to note that oxygen evolution of the everted mangrove thylakoid vesicles showed essentially the same salt-response curve as had previously been demonstrated for mangrove thylakoids. Thus, their salt tolerance is not an effect due to an extremely low chloride permeability of the mangrove thylakoid membrane which would protect the water-oxidation site from the high chloride concentrations.

The mangrove inside-out thylakoid vesicles showed the same partition in the aqueous polymer two-phase system as the corresponding spinach vesicles. The phase partition technique separates membranes according to difference in surface properties [25] and can therefore be used to sense subtle changes in a membrane surface related to development, adaptation, metabolic stages and pathological variations [25]. The great similarity in partition behaviour between spinach and mangrove vesicles therefore suggests that the salt tolerance of the mangrove thylakoids is not the result of gross adaptative changes to their membrane surface charge or hydrophobicity. The salt tolerance is probably due to more specific changes in (a) certain protein(s).

Subjecting chloroplasts which are derived from salt-tolerant plant species to low salt and low osmolar media must result in osmotic rupture of the membranes because only such processes would allow polypeptides to be lost from the inner thylakoid membrane. Ball et al. [26] reasoned that isolation of intact chloroplasts from salt-tolerant species would depend on maintaining the osmotic strength of media at a level consistent with that prevailing in the intracellular environment. Indeed, mangrove chloroplasts isolated and kept in buffer containing 1 M sorbitol (osmotically equivalent to seawater) did not require added chloride for O_2 evolution [26] nor did they lose their 23 or 16 kDa proteins [23]. The osmoticum acting in vivo is as yet unknown but could be NaCl or glycine-betaine [30,31] or mixtures thereof. It is conceivable that the polypeptides extrinsic to the thylakoid membrane on both sides, such as the 23 and 16 kDa proteins of the O_2-evolving complex, plastocyanin, ferredoxin and CF_1, have altered structures which confer salt tolerance or high osmotic pressure tolerance on the thylakoid membrane.

The correlation between oxygen-evolution activity at low chloride concentrations and the presence of the 23 and 16 kDa proteins indicated the possibility of chloride-binding sites on these proteins [23]. Moreover, readdition of the 23 kDa protein alone decreased the chloride requirement for oxygen-evolution approx. 50-fold. Further evidence for the 23 kDa protein being associated with chloride was obtained from the preillumination experiments with both mangrove and spinach membranes. The 23 kDa protein protected efficiently against inhibition of electron transport during illumination of thylakoids in a salt-depleted medium. Whether this inhibition is in fact photoinhibition [32] remains to be established. In the absence of the protein, protection against this inactivation could only be obtained by raising the chloride concentration of the medium. Taken together, these observations independently provide strong evidence for a regulatory role of the 23 kDa protein in chloride binding to the O_2-evolving centre. Support for this view has recently been presented by Nakatani [33] who showed that the 23 and 16 kDa proteins in isolated Photosystem II particles could be replaced by high concentrations of NaCl. The mechanism by which the 23 kDa protein increases the affinity of the oxygen-evolving complex for chloride is not clear. The 23 kDa protein itself may carry high affinity sites for chloride. Alternatively, the presence of the 23 kDa protein may indirectly influence chloride binding by inducing some conformational change in the actual water-oxidation site. Our data show clearly that without the 23 kDa protein chloride binding to the oxygen-evolving complex must be quite weak.

Recent studies on Photosystem II particles [20,21] show that the 23 and 16 kDa proteins can also be replaced by calcium. Ghanothakis et al. [22] have convincingly shown that one or both proteins interact with the calcium required for oxygen evolution. Probably, the chloride and calcium effects correlated with the 23 kDa protein are related so that both results demonstrate essential regulating roles for the 23 kDa protein in optimizing oxygen evolution. High concentrations of chloride not only replace the 23 kDa protein in oxygen evolution but also drastically change the pH optimum for the reaction. In the presence of the protein, the pH optimum is 6.3, while in its absence or in the presence of high chloride it is 7.0. The reason for this effect is not understood but may be due to pK_a changes of the manganese-carrying protein(s) [15] and/or may be related to the apparent existence of a special proton compartment around the water-oxidation site [34,35].

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