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The chloride and calcium requirement of photosynthetic water oxidation: effects of pH

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Photosystem II membranes were isolated from chloroplast thylakoids of *Phytolacca americana* and *Spinacia oleracea*. After removal of activating Cl^- or Ca^{2+}, the pH dependence of the response of the membranes' water-oxidizing activity to a readdition of these ions was analyzed. The results were as follows: (1) the activation of water oxidizing activity by added Cl^- was controlled by groups having not a pK_a around 6 as previously concluded, but a $pK_a < 5$ regardless of the presence of the extrinsic 23 and 17 kDa polypeptides; (2) at pH 5, the apparent K_m for Cl^- binding was well below 1 mM even in the absence of those polypeptides; (3) the binding of one H^+ accompanied the acquisition of one activating Cl^-, but the H^+ requirement may have been less after removal of the two polypeptides; (4) the number of Cl^- anions needed per active enzyme complex appeared to decrease with decreasing pH; (5) for Photosystem II membranes depleted of their 17 and 23 kDa extrinsic polypeptides, an existence of high- and low-affinity Ca^{2+}-binding sites with apparent K_m values of around 60 μM and above 1 mM was confirmed; (6) the high- and low-affinity binding sites were found to be interconvertible with the low-affinity sites dominating at low pH. As an alternative to a binding of the activating Cl^- to histidines with an unusually low pK_a, it is suggested that binding occurs on positively charged groups, the availability of which is controlled by the protonation state of neighboring anionic groups, presumably carboxylates. The positively charged Cl^- binding entities may either be protonated amino or imino groups or, as favored by others, the Mn cluster at the active site of the water oxidase. The extrinsic 17 and 23 kDa polypeptides may serve as Cl^- buffer for the catalytic site of the water oxidase. While the polypeptides are not essential for providing high affinity Ca^{2+} binding sites, the 23 kDa species is nevertheless needed to shield bound activating Ca^{2+} from the slightly acid pH of the thylakoid lumen during in situ electron transport.

Abbreviations: Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; Chl, total chlorophyll; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDS or SDS, lithium or sodium dodecylsulfate, respectively; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; PS II, Photosystem II.

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Introduction

In spite of our growing knowledge about the composition and operation of the water-oxidizing complex in Photosystem II (PS II) of chloroplasts, we do not well understand the specific functions of many of its constituents. This is true, for example, for the two small extrinsic polypeptides of molecular masses around 17 and 23 kDa, and the

inorganic cofactors Cl^- and Ca^{2+} (for reviews, see Refs. 1–5). One function of the two polypeptides apparently is to retain the natural complements of Cl^- and Ca^{2+} of PS II at the water-oxidizing site. Thus, so long as the polypeptides are in place, almost optimal oxygen evolution activity may be displayed by PS II preparations even in media free of added Cl^- and Ca^{2+}.

According to the available experimental evidence, the ready loss of Cl^- from the water-oxidizing site after removal of the 17 and 23 kDa polypeptides can be attributed to a considerably lowered affinity of the anion to its sites of interaction. Maximal O_2-evolution activities of polypeptide-depleted membranes, therefore, depend on the presence of at least 20 mM NaCl in the assay medium, while after reconstitution of the polypeptide the requirement is 10-times lower [6–9].

From the pH dependence of the Cl^- requirement of PS II membranes, we had concluded previously that Cl^- binding occurred on (amino?-) groups with a pK_a around 6 [8]. However, subsequent experiments [10,11] raised the possibility that our earlier data were confounded by a dissociation of the 23 kDa polypeptide during our Cl^- depleting treatment, and during assays at pH > 6.8. It had become necessary, therefore, to repeat the analyses and extend them to cover the pH range below 5.8. As reported in this article, the new results were incompatible with the concept of Cl^- binding groups of $pK_a \approx 6$. Instead, they suggest a much lower pK_a for the groups controlling the reactivation of water oxidation by Cl^-, regardless of the presence or absence of the extrinsic 17 and 23 kDa polypeptides. This finding puts considerable restrictions on any speculation about the way catalytically active Cl^- interacts with the water-oxidizing complex of chloroplasts.

In comparative studies, we also analyzed the pH dependence of the relations between the water-oxidizing complex and its cofactor Ca^{2+}. Functional Ca^{2+} is known to be essentially unextractable when the 23 kDa polypeptide is in place [12,13] and to remain so tightly bound after removal of the polypeptide that treatments with light [14–16], or with chelators like EGTA or the ionophore A23187 [12,15,17] are necessary to create a Ca^{2+}-deficient state. The tenacity with which PS II preparations hold on to their Ca^{2+} even after detachment of the 23 kDa polypeptide suggests an association of rather high affinity. Yet, Yocum and coworkers [12] observed and recently affirmed [13,18] that the Ca^{2+} requirement of depleted PS II membranes can be satisfied by submillimolar concentrations of the added cation only after the 23 kDa polypeptide has been reconstituted. They suggested that this polypeptide provides the conditions necessary for a high-affinity interaction of Ca^{2+} with the water-oxidizing site. Work in other laboratories, however, has established that a removal of the 23 kDa polypeptide did not eliminate the high-affinity binding sites from all water-oxidizing complexes [15,17].

Our own investigations confirmed the Ca^{2+} retaining role of the 23 kDa extrinsic polypeptide and the presence of at least two types of Ca^{2+} binding sites on polypeptide depleted membranes. High-affinity interactions of Ca^{2+} were found to be more prevalent at pH > 6, while low-affinity interactions predominated at pH $\lesssim$ 5.5. It is suggested that a low affinity for Ca^{2+} may be the consequence of protonation-induced structural changes of the protein components of the water-oxidizing complex. Differences in the susceptibility to such changes may explain the inconsistencies between the reported affinities of PS II membranes for activating Ca^{2+}.

Materials and Methods

All experiments were performed with PS II-enriched membranes isolated essentially according to Berthold et al. [19] with Triton X-100 from chloroplast thylakoids of wild-growing pokeweed (*Phytolacca americana* L.) or market spinach (*Spinacia oleracea* L.). The membranes were washed and stored at −60 °C in 0.5 M sucrose containing 1 mM NaCl and 10 mM Na-Mes (pH 6.2) at 5–10 mg Chl/ml [8]. The Chl *a*/Chl *b* ratio of our preparations was 2.2 ± 0.1.

Unless stated otherwise, removal of the 23 and 17 kDa polypeptides was accomplished with only occasional exposure to room light at 0 °C by treatment for 30 min with 1.5 to 2 M NaCl in 0.4 M sucrose buffered with 40 mM Na-Mes at pH 6.4, and containing 1 mM phenylmethylsulfonylfluoride [20]. After sedimentation by centrifuga-

tion, the membranes were washed three times and stored like the controls.

The quality of our PS II preparations and the success of the polypeptide removing procedure was checked by SDS-polyacrylamide gel electrophoresis performed at 5 °C. We followed the procedure of Chua and Bennoun [21] but used as reservoir buffers 0.1% SDS in 25 mM Tris-glycine (pH 8.3) [22] and included 3 M urea in the gels from 13.5 or 12.5% acrylamide plus 0.27 or 0.34% *N,N'*-methylenebisacrylamide for *P. americana* or *S. oleracea* membranes, respectively. LDS was used in place of SDS for for protein solubilization.

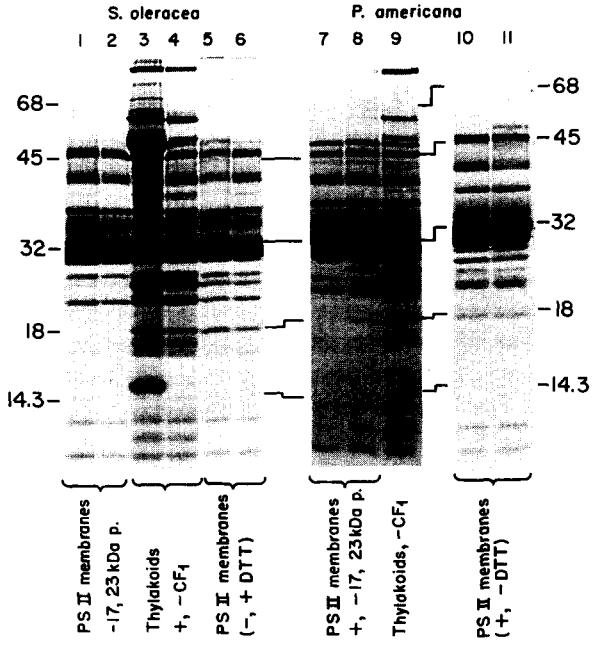

Fig. 1. Protein profiles as determined by LDS-polyacrylamide gel-electrophoresis of thylakoid preparations and PS II membranes isolated from chloroplasts of *P. americana* and *S. oleracea*. Lane 1: *S. oleracea* membranes depleted of the 17 and 23 kDa polypeptides by treatment with 2 M NaCl; lane 2: same, but polypeptide depletion during dialysis according to Ref. 10; lane 3: *S. oleracea* thylakoids; lane 4: same, but depleted of CF_1 by treatment with 1 mM EDTA in a low-ionic-strength medium; lane 5 and 6: *S. oleracea* membranes, solubilized in the absence and presence of 50 mM dithiothreitol, respectively, lane 7: *P. americana* membranes, depleted of the 17 and 23 kDa polypeptides by treatment with 2 M NaCl; lane 8: same, polypeptides not removed; lane 9: *P. americana* thylakoids, CF_1 removed as for lane 4; lanes 10 and 11: *P. americana* membranes solubilized in the presence and absence of dithiothreitol, respectively (different electropherogram, but same preparation, as lanes 7–8).

Coomassie Brilliant Blue-stained protein profiles of typical preparations before and after removal of the 17 and 23 kDa polypeptides are shown in Fig. 1, and compared with electropherograms of intact thylakoids. One peculiarity of the PS II membranes from *P. americana* thylakoids was a dark-staining band at approx. 50 kDa which was, with the same preparations, seen in some but not all electropherograms. Since its appearance could be guaranteed, even with spinach membranes, by omitting the customarily added 50 mM dithiothreitol from the solubilization medium (see Fig. 1), it probably was due to an association of two, yet unidentified, polypeptide molecules of the PS II complex.

Cl^- depletion of PS II membranes carrying their 17 and 23 kDa polypeptides was accomplished as described elsewhere [8,23] by incubating a Cl^--free assay suspension of untreated preparations at pH 10 for a duration of 7–10 s. Removal of Ca^{2+} was accomplished either by a 30 s exposure of the assay suspension of polypeptide-depleted PS II membranes to pH 5 in the presence of 100 μM EDTA or EGTA, or by the method of Ghanotakis et al. [12], according to which previously polypeptide-depleted, or control, membranes are dialyzed at 0.5 mg Chl/ml against 0.4 M sucrose containing 2 M NaCl, 40 mM Mes (pH 6.0) and 5 mM EGTA. The dialyzed preparations were washed once with an identical medium and subsequently with our standard wash medium after having been supplemented with 1 mM EGTA.

O_2 evolution activity was measured at 25 °C with a Clark-type oxygen electrode (Yellow Springs Instrum.) in 3 kW/m^2 red light [8] with membranes equivalent to 6–8 μg Chl/ml using an assay medium that always contained 0.4 M sucrose. 500 μM phenyl-*p*-benzoquinone served as electron acceptor and Cl^- was usually supplied as NaCl. Flash illuminations were possible with an EG&G Model 2P-3 flash lamp.

The standard incubation time for reconstitutions with Cl^- or Ca^{2+} was 3 min which was found to yield activities that were more than 90% of the maximally attainable under the conditions. All postpreparative treatments and incubations of the PS II membranes were done in very dim light at room temperature (approx. 25 °C) unless indicated otherwise, while the stock suspensions were

kept on ice. Dark adaptations of the O_2-evolving apparatus was accomplished by overnight storage of the stock suspension of PS II membranes on ice in a closed Dewar flask; subsequent handling was done in dim green safelight.

Results

The pH dependence of the activation of Cl^--depleted water-oxidizing complexes by added Cl^-

When, in our previous studies [8,24], we estimated the apparent K_a of the functional groups controlling the binding of the cofactor Cl^- to Cl^--depleted PS II membranes, we were not aware that the 17 and 23 kDa extrinsic polypeptides might dissociate during the Cl^--depleting incubation at pH 10 and in assay media of a pH close to 7 [10,11]. For a reassessment of those earlier results, we decided to extend the previous analyses towards the acid region. PS II membranes from *Phytolacca* were particularly suited for these investigations because, as shown elsewhere [11], their extrinsic polypeptides do not dissociate as readily at an elevated pH as those of other preparations.

Determinations of the Cl^- requirements of photosynthetic O_2 were made in media of a pH between 7.4 and 4.3 with PS II membrane preparations carrying, or devoid of, their 17 and 23 kDa extrinsic polypeptides. As in our earlier studies, we analyzed our data in double reciprocal plots of O_2 evolution rates vs. Cl^- concentrations. This allowed us to estimate, by extrapolation, the apparent K_m for the interaction of activating Cl^- with the water-oxidizing complex [8,25]. One problem we encountered while using this approach was the finite rate of O_2 evolution that remained after the Cl^--depleting treatment. So long as this rate was less than about 15% of the rate measured at optimal Cl^- concentrations, it could be neglected. That was the case when the medium pH had been above 5.8, and in all experiments with membranes devoid of the 17 and 23 kDa polypeptides. At lower pH, however, the residual activity was considerably higher, reaching

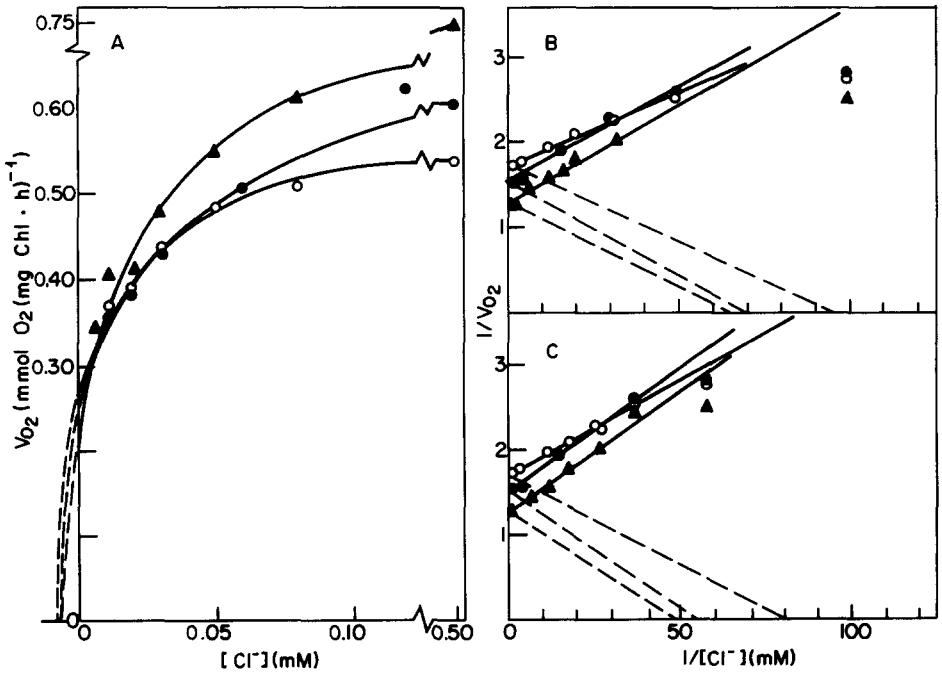

Fig. 2. Dependence of the O_2 evolution activity of Cl^--depleted *Phytolacca* PS II membranes on the concentration of added Cl^- in media of pH $\leq$ 5.2. Media were buffered with 40 mM Na-succinate and contained 35 μg Chl/ml. (A) Plot of O_2 evolution activity vs. [NaCl]; dashed line extrapolation: see text and Ref. 25. (B) Data of (A) presented in double reciprocal plots. (C) Same, but [Cl^-] data corrected for the extrapolated residual Cl^- responsible for the activity without added NaCl (dashed lines in B and C, mirror image of extrapolation of lines towards abscissa to estimate K_m^{-1}). ○, pH 4.3; ●, pH 4.75; ▲, pH 5.2.

about 30% at pH 5.2, and becoming higher yet below pH 5. This is shown in Fig. 2. In an earlier study with intact thylakoids, Kelley and Izawa [25] extrapolated the rate vs. concentration curve to estimate the Cl^- concentration that might have supported the residual activity, and adjusted the concentration value of the added Cl^- accordingly. Their approach is justified if one accepts the notion that Cl^- is, in fact, an essential activator of the photosynthetic water-oxidizing mechanism. Fig. 2 demonstrates the application of the correction procedure of Kelley and Izawa. It can be seen that the correction had not much effect on the estimated K_m, even with an almost 50% residual rate. Thus, the error margin from the uncertainty of the extrapolation was acceptable. It is noteworthy that a not very different K_m value (approx. 30 μM Cl^-) was estimated for pH 4.3 and 4.75 when $(V - V_{residual})^{-1}$ was plotted against $[Cl^-]^{-1}$, i.e., when Cl^- was treated as a nonessential activator and, hence, the residual rate, $V_{residual}$, was attributed to a Cl^--independent activity of the water-oxidizing enzyme.

From the results of Fig. 2, and the data of Fig. 3 from analyses in media of a higher pH, it can be seen that the Cl^- requirement decreased progressively down to pH < 5. The apparent K_m around that pH turned out to be about 20 μM when the 17 and 23 kDa polypeptides were still attached, but was approximately 10-times higher after their removal. This again confirmed the dramatic influence of the extrinsic polypeptides on the response of the water-oxidizing complex to added Cl^-. Nevertheless, even in the absence of the polypeptides, the Cl^- requirement could become quite low and 'physiological'.

A severalfold lower Cl^- requirement at pH 5, than at pH 6, clearly was incompatible with our earlier conclusion [8] that Cl^- binding was contingent upon the protonation of some functional group with a pK_a around 6. This view had been based on an extrapolation of the straight line

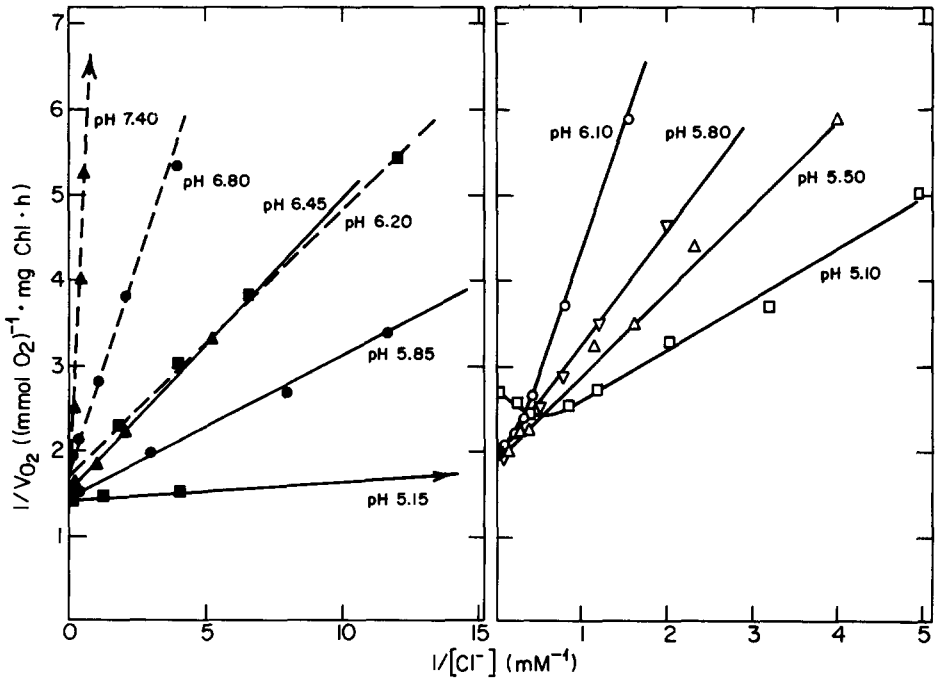

Fig. 3. Double reciprocal plots of Cl^- dependence vs. O_2 evolution activity of *Phytolacca* PS II membranes with, or lacking (right panel), the extrinsic 17 and 23 kDa polypeptides. Buffering with 40 mM Na-Mops at pH 7.4 and 6.8, Na-Mes at 5.1 to 6.45; ——— and ------: two different experiments. Buffers of assay media for polypeptide-depleted membranes supplemented with $Ca(OH)_2$-neutralized buffer to give 2 mM Ca^{2+}. For polypeptide-carrying membranes, rates were reduced by the Cl^- depletion treatment to between 33% (pH 5.1) and 5% (pH 7.4) of the fully reactivated rate. Arrow indicates that additional data points beyond scale boundary were available for the plot.

obtained in a plot of apparent K_m values vs. $1/[H^+]$ for the range pH 5.9–7.1. Theoretical considerations [26] allow to attribute a linear relationship in such a presentation to an ordered sequential binding of an H^+ and the activating Cl^-.

Since the range of H^+ concentrations covered by our new data spanned three orders of magnitude, their presentation in a K_m vs. $1/[H^+]$ plot was impractical. Instead, we chose logarithmic coordinates and plotted pK_m (apparent) vs. pH as proposed by Dixon [27] for analyses of the mechanism underlying the pH dependence of the interactions of enzymes with their substrate (or activator). Simple straight lines are expected whenever the range of the pH tested is removed from the pK_a of a protonatable group that is critical for substrate binding. The theoretical concepts behind the pK_a vs. pH plots also include the situation in which one protonation event has to precede the binding of an activator, i.e., exactly the condition that produces a straight line relationship in the plot K_m vs. $1/[H^+]$ mentioned above. In fact, straight lines in the latter plot would be expected to yield straight lines in a pK_a vs. pH plot with a slope -1. This follows when the equation

$$K_m(\text{app}) = K_m(1 + K_{H^+}/[H^+]) \quad (1)$$

underlying the K_m(app) vs. $1/[H^+]$ plot is approximated for $[H^+] \ll K_{H^+} = K_a$ of the involved protonatable group to become

$$K_m(\text{app}) = K_m \cdot K_{H^+} \cdot 1/[H^+] \quad (2)$$

and, after taking the logarithm,

$$pK_m = C - \text{pH} \quad (3)$$

Fig. 4 shows that most of the data of Figs. 2 and 3 could, indeed, be approximated by a straight line in a plot of pK_m(app.) vs. pH. As predicted for a dependence of Cl^- binding on a single protonation event, the line connecting the K_m values determined for membranes carrying their 17 and 23 kDa polypeptides (plot 'a', filled symbols) had a slope of approx. -1. At pH 5, the straight line had an abrupt break as a limiting K_m was approached. Of course, the K_m values for pH < 5 had been arrived at by applying to the experimental data a considerable correction, and they cover a pH range in which optimal activities could no longer be obtained. However, even a superficial inspection of the relevant rate vs. concentration curves in Fig. 2 confirms a very low sensitivity of the Cl^- requirement to the medium pH between 5 and 4.3. We suggest, therefore, that the pK_a of the groups controlling Cl^- binding lies between 4 and 5.

In Fig. 4, plot 'b', the pK_m values determined for the polypeptide-depleted membranes are approximated by a straight line with a slope of -0.7. Data from experiments other than those documented in Fig. 3 have been included in the graph to support further the observed deviation of the slope from unity. It should be noted that some of the relevant data were gathered in the presence of Ca^{2+}, and others in its absence, yet all follow essentially the same line. This suggests that Ca^{2+} did not alter the response to Cl^- although it did, as was expected from the work of Yocum's group [12], increase the V_{max} (not shown).

Additional support for a non-unity slope of the pK_m (Cl^-) vs. pH plot for polypeptide depleted PS II membranes came from independent work by

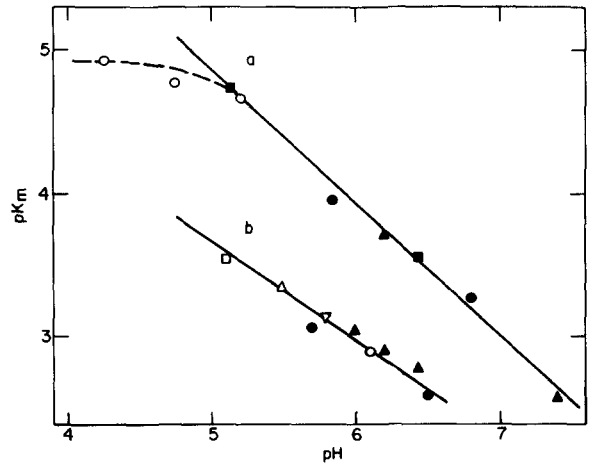

Fig. 4. Plots of pK_m(app) vs. pH for Cl^- binding as estimated from double reciprocal plots of the kind shown in Figs. 2 and 3. (a) Closed symbols: data from the experiment of Fig. 3 with polypeptide carrying *Phytolacca* membranes; open circles, data from Fig. 2. (b) Open symbols: data from experiment with the polypeptide-depleted *Phytolacca* membranes of Fig. 3. ▲, data from same preparation determined in the absence of Ca^{2+}; ●, data from polypeptide-depleted *Spinacia oleracea* membranes (Ref. 24).

Itoh and Uwano [28]. The authors have published a plot with a slope −0.8 for the pH dependence of the Cl^- requirement of PS II membranes from which Cl^- had been removed by a treatment with SO_4^{2-} at pH 7.5. According to our experience [10,11], such a Cl^--depleting treatment would cause a dissociation of the extrinsic 17 and 23 kDa polypeptides. That this actually happened in the experiments of Itoh and Uwano, is suggested by their rather high K_m values for the reactivation by Cl^- (almost 1 mM at pH 6), and by the low activity of their preparations at at pH < 6 (cf. Fig. 6 below).

Adopting Dixon's interpretation of the double log plots pK_m vs. pH [27], it would appear that Cl^- binding to water oxidase complexes depleted of their 17 and 23 kDa polypeptides may be linked to the binding of less than 1 H^+. The apparent pK_a of the groups controlling Cl^- binding, on the other hand, appeared to be below 5, just as in the case of the fully assembled water-oxidizing complexes.

A possible pH dependence of the amount of activating Cl^- needed by the water-oxidizing complex

As was mentioned above, the residual water-oxidizing activity remaining after Cl^- 'depletion' became higher with decreasing pH of the assay medium. One obvious explanation of this phenomenon was that the lowered Cl^- requirement allowed a greater degree of reactivation to occur by contaminating Cl^-. This may have been true around pH 5, and below. But our experiments in that pH range also suggest that the level of contamination in the assay media must have been 20 μM or less, hardly enough to cause measurable differences in residual rates at pH 6 and above. Thus, we wondered whether the medium pH perhaps affected not only the binding constant(s) for Cl^-, but also the actual number of activating Cl^- needed by each water-oxidizing complex.

To test this supposition, the following argument was used: if the O_2-evolution activity were an invariable measure of the amount of bound Cl^-, one should be able to activate depleted centers with limiting concentrations of the anion at pH 5 and measure identical activities at that pH and after raising it, provided the maximal possible activities remain the same and Cl^-, once bound, is not readily lost again. These conditions were met between pH 5 and 6.5 (Ref. 8; see also below). Table I contains data from experiments in which a partial activation of dark-adapted PS II membranes had been accomplished with very low concentrations of Cl^- at pH 5.1, and activities were measured either at pH 5.1 or after a pH shift to 6.2. It can be seen that, although the activity at pH 6.2 was higher than that obtained when Cl^- had been added after the pH shift, it was much lower than that measured at pH 5.1. We considered the possibility that perhaps the rates at pH 6.2 were lower because the activating Cl^- binding at pH 5.1 did not occur until the actinic light was turned on. However, as the relevant data of Table I show, a flash illumination of our dark-adapted PS II membranes prior to the pH shift did not affect the rate subsequently measured at pH 6.2. A brief period of continuous illumination actually caused a lower activity, presumably due to photoinhibition of the partially Cl^--deficient preparation [14,29,30].

One aspect of concern about the data of Table I may be the different ionic strengths provided by the buffers we used to attain the two pH values.

TABLE I

THE DEPENDENCE OF THE 'Cl^- STATUS' OF PS II ON pH

After Cl^--depleting exposure to pH 9.8 with 2.5 mM Na-Caps, the suspension of *P. americana* PS II particles was brought to pH 5.1 with the appropriate amount of 1 M Mes, giving about 10 mM Mes in the solution. Cl^- was added immediately therafter, or after a further pH change to pH 6.2 with approx. 20 mM Na-Mes (pH 6.85). Data of the last two columns represent experiments in which a 1.5 min incubation with Cl^- at pH 5.1 preceded the pH change to 6.2 for activity measurement at that pH. Preillumination at pH 5.1 with one flash or, in an experiment of another day (bottom line in table), with 10 W/cm² red light for 20 s.

Added [Cl^-] (mM)	O_2-evolution activity (μmol O_2 per mg Chl per h)			
	pH 5.1	pH 6.2	pH 5.1 → pH 6.2 not preill.	preill.
0	130	100	–	–
0.02	250	125	165	180
0.04	320	150	220	220
16	590	625	–	–
0.04	345	190	280	250

However, it was ascertained in separate tests that, at pH 5.1, the buffer concentration could be raised fivefold without a significant change in activity, while the rate measured at pH 6.2 was affected only when the buffer concentrations were decreased significantly below the range used in the experiments. Furthermore, data are included in Table I, showing that, under the experimental conditions chosen, the maximal rates attainable at either pH 5.1 or at pH 6.2 were almost identical. Thus, the change of the ionic strength of the media during the pH jump contributed little to the observed rates.

In order to obtain an independent confirmation of the apparent pH dependence of the degree of activation with a given amount of acquired Cl^-, we performed an experiment in which we allowed Cl^- to dissociate from the membranes at pH 6.8, and measured the remaining activity after pH shifts to 6.2 and 5.2. Fig. 5 shows that higher rates were sustained at the lower pH, consistent with the data described above. The data of Fig. 4 could not be attributed to a greater degree of reactivation by the liberated Cl^- after lowering the pH to 5.2 because much lower rates were measured at that pH in the same type of medium after a more complete Cl^- dissociation (e.g., Table I). Fig. 5 also documents that the activity of the partially Cl^--depleted preparations after the pH shift to pH 6.2 did not decline during a 5 min incubation prior to the measurement. This proved the stability of the Cl^- status of our preparations at pH 6.2 that was a precondition for the reliability of the data in Table I.

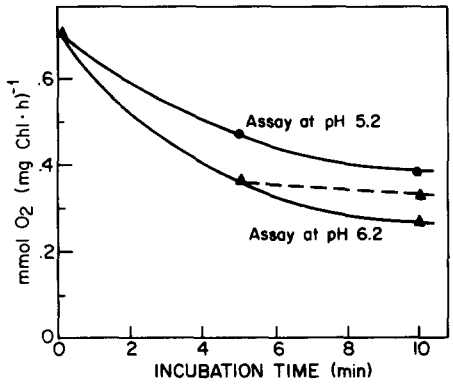

Fig. 5. O_2 evolution activity of PS II membranes after incubations in a Cl^--free medium buffered at pH 6.8 with 5 mM Na-Mops. After times indicated on the abscissa, unneutralized Mes was injected to adjust the pH to the desired value. Following injection of the electron acceptor, the activity was determined. Dashed line describes an experiment in which a 5 min incubation in the dark followed the pH change.

The pH dependence of the activation of polypeptide-depleted PS II membranes by Ca^{2+}

In our studies on the consequences of a depletion of the 17 and 23 kDa polypeptides from PS II membranes, we noted not only a variable degree of decreased O_2-evolution activity, but also a greater sensitivity to a lowering of the medium pH than was typical for undepleted preparations. This is shown in Fig. 6 for preparations isolated from pokeweed and spinach. In the figure, data are also included which document that the 'acid sensitivity' of the polypeptide-depleted preparations could be diminished by including Ca^{2+} in the reaction medium. Ca^{2+} did not, however, alter the activities of undepleted control membranes, consistent with the concept of a Ca^{2+}-retaining function of the 23 kDa polypeptide [12,18]. We also ascertained (not shown) that provision of the 23 kDa polypeptide alone reestablished the obstruction of the Ca^{2+} binding site which Ghanotakis et al. [12] have described.

It was logical to attribute the observed Ca^{2+} effect to a labilization of the bound cation in the polypeptide-depleted membranes when the pH was lowered. In accordance with such a view, the data of Table II show that the portion of Ca^{2+}-dependent water-oxidizing activity was increased when the polypeptide depleted PS II membranes were incubated at pH 5 in the presence of EDTA before being assayed at pH 6. Light was not required for a removal of Ca^{2+} at pH 5, but a light exposure increased somewhat the Ca^{2+} requirement of preparations kept at pH 6, consistent with observations of others [14,15]. We established, furthermore, that artificial electron donors to PS II like NH_2OH or tetraphenylboron restored fast fluorescence induction kinetics in our Ca^{2+}-depleted membranes (not shown). This test confirmed that the Ca^{2+}-depleting treatment impaired reactions close to, or in, the water-oxidizing complex, and did not affect the operation of the PS II reaction center.

Even though an exposure of polypeptide-de-

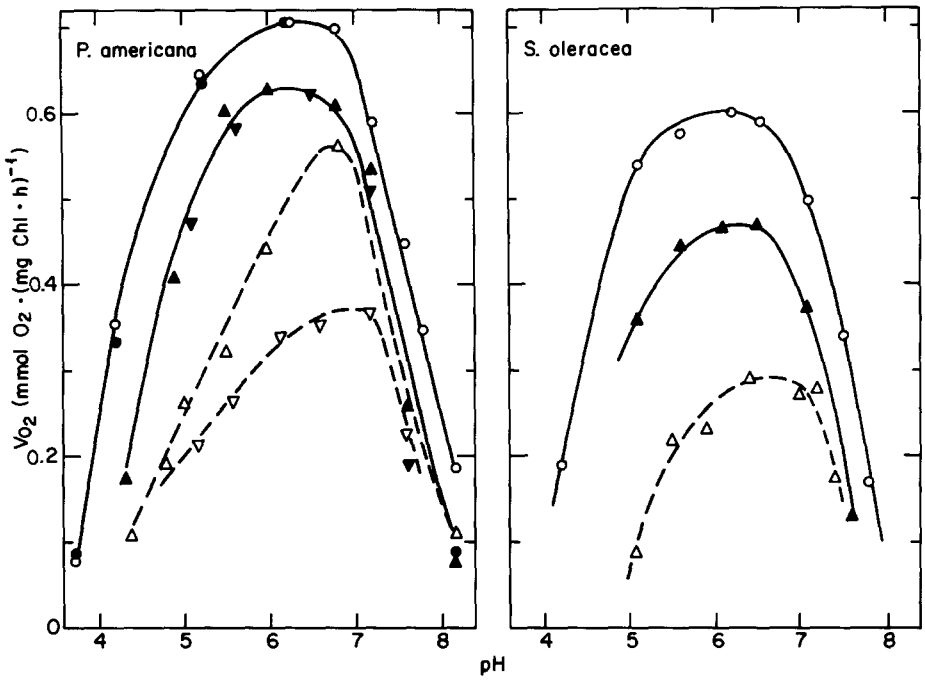

Fig. 6. pH dependence of O_2 evolution activity of PS II membranes isolated from *Phytolacca americana* or *Spinacia oleracea* chloroplasts. 35 mM NaCl was present, and 50 mM buffer: Na-Hepes for pH 7.5; Na-Mops between pH 6.7 and 7.5; Na-Mes between 5.1 and 6.6; Na-succinate for pH < 5. Incubation before measurement 2 min. ○, controls; ●, same, in presence of 2 mM $CaCl_2$; △, preparations depleted of the 17 and 23 kDa polypeptides; ▲, same, but in presence of 2 mM $CaCl_2$. ▼▽: a separate experiment with a different polypeptide-depleted preparation of *P. americana* in presence and absence of $CaCl_2$, respectively; for better comparison, the measured values were normalized by multiplication with 1.08, the ratio of the optimal activity of ▲ over that of ▼.

TABLE II

INDUCTION BY LOW pH OF A Ca^{2+}-DEFICIENT STATE IN PS II MEMBRANES DEPLETED OF THEIR 17 AND 23 kDa POLYPEPTIDES

Polypeptide-depleted *P. americana* PS II membranes (7 μg Chl/ml) were incubated for 45 s in a medium buffered in the absence of added Ca^{2+} and Cl^- with 10 mM Na-Mes at pH 6 or 5.1 and containing 100 μM EDTA. Cl^- was then provided to a total of 20 mM as NaCl or, when applicable, as 16 mM NaCl and 2 mM $CaCl_2$. The pH change was effected by injection of Na-Mes (pH 6.85). Dark-adapted preparations were used unless illumination regime is indicated as 'dim'. Flash illuminations were at the beginning and the end of the incubation period.

pH of incubation	Illumination during incubation	pH during assay	Ca^{2+} in assay	Rate (μmol O_2 per Chl per h)
6.1	dim	6.1	–	385
5.1	dim	6.1	–	155
5.1	dim	6.1	+	555
6.0	–	6.0	–	363 ± 22
6.0	2 flashes	6.0	–	296 ± 46
6.0	2 flashes	6.0	+	583
5.1	–	6.0	–	183
5.1	–	6.0	+	615

pleted PS II membranes to pH 5 created a considerable degree of Ca^{2+} deficiency, we preferred to use the Ca^{2+} depletion protocol of Ghanotakis et al. [12] (see Materials and Methods) because it generally gave more reproducible results, and was more easily applied to large amounts of membranes. The dependence of the O_2-evolution activity of such preparations on the concentration of added Ca^{2+} was determined as described earlier in this article for the experiments with Cl^-, and the obtained data were corrected and displayed in a corresponding fashion.

Fig. 7 shows data obtained with *Phytolacca* membranes which confirmed the apparent heterogeneity of Ca^{2+} binding sites described by Boussac et al. [15] and by Cammarata and Cheniae [17]. It is also evident that a decrease of the medium pH not only led to generally lower affinities for Ca^{2+}, but also changed a predominance of high-affinity binding sites to a greater prevalence of low-affinity sites. This was quite consistent with the pH profiles of the water-oxidizing activities in the presence and absence of Ca^{2+} shown in Fig. 6. For the high affinity interaction at pH 6.4 one can determine an apparent K_m of approx. 70 μM, while simulations suggested several millimolars for the K_m of the low affinity binding at pH 5.65, in

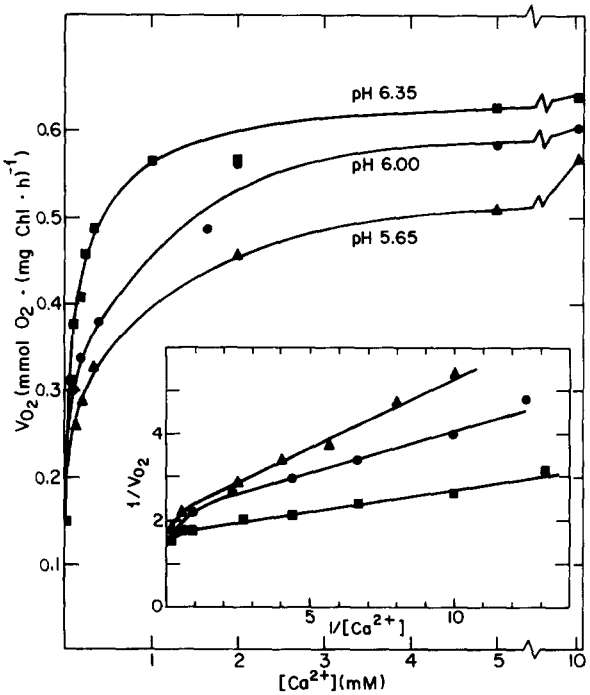

Fig. 7. Ca^{2+} requirement of PS II preparations from *Phytolacca americana* devoid of the 17 and 23 kDa polypeptides at different pH after Ca^{2+} depletion by dialysis against a EGTA-containing medium [12]. Medium contained 25 mM Cl^- supplied as mixture of NaCl and $CaCl_2$, and was buffered with 40 mM Na-Mes. Inset: double reciprocal plots of corrected data (see text) from main panel.

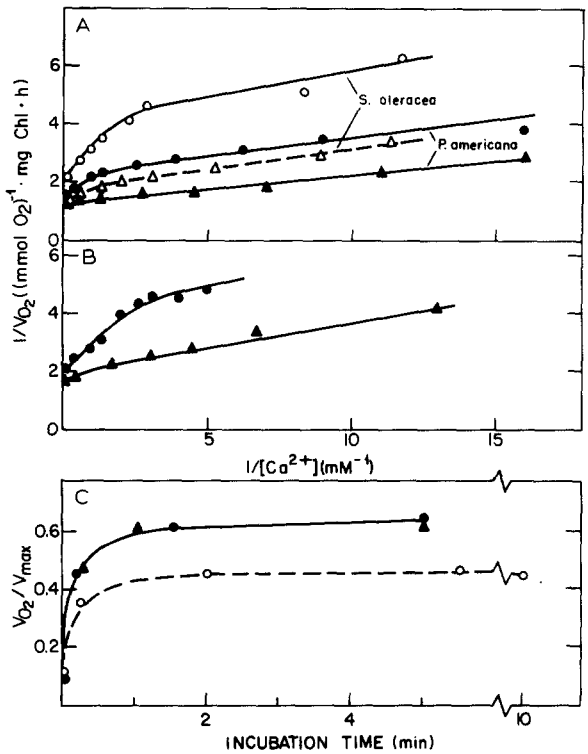

Fig. 8. Demonstrations of heterogeneity of Ca^{2+} binding sites in PS II membranes from *Phytolacca americana* and *Spinacia oleracea* depleted of their 17 and 23 kDa polypeptides. Media buffered with 40 mM Na-Mes, and 25 mM Cl^- was provided as mixtures of NaCl and $CaCl_2$. (A) Double reciprocal plots of Ca^{2+} dependence of O_2 evolution activity of *P. americana* (▲, ●) and *S. oleracea* (△, ○) membranes; Ca^{2+} depletion by dialysis against an EGTA-containing medium [12]; ▲, △: pH 6.4; ●, ○: pH 5.6. (B) Double reciprocal plot of Ca^{2+} dependence of O_2 evolution activity for polypeptide depleted *P. americana* membranes exposed to pH 5.1 for 1 min in the presence of 100 μM EGTA. Activity measurement either at pH 5.1 (●) or after pH change to pH 6.2 (▲). (C) O_2 evolution activity for *P. americana* (●, ▲) and *S. oleracea* (○) membranes after incubations (0.7 mg Chl/ml) at pH 6.2 in the presence of 2 mM $CaCl_2$, and subsequent 100-fold dilution for assay with a solution of 0.4 M sucrose, 40 mM Na-Mes (pH 6.2), 20 mM NaCl and 500 μM EGTA (except for ▲ when EGTA was omitted). Rates were given as fraction of rate measured with 10 mM $CaCl_2$ in the assay medium.

good agreement with values reported by Boussac et al. [15] for spinach, and Cammarata and Cheniae [17] for wheat membranes. The latter authors noted that their preparations from wheat typically displayed a greater abundance of high-affinity sites than PS II membranes from spinach. As Fig. 8A reveals, we found this to be true also for membranes isolated from pokeweed thylakoids.

Since Cammarata and Cheniae [17] had estimated a requirement of 2–3 Ca^{2+} per PS II unit, it was possible that the pH effect on the relative abundance of the alleged high- and low-affinity sites was due to a removal of different pools of Ca^{2+} in media of different pH. However, Fig. 8B provides evidence that the low-affinity response at pH 5 could be converted to one of high affinity by simply raising the pH to 6.2.

When bound to a high-affinity site, activating Ca^{2+} is resistant to removal by the chelator EGTA [17]. Thus, the relative abundance of such sites can be estimated by incubating polypeptide-depleted, Ca^{2+}-deficient membranes in the presence of millimolar concentrations of the cation, and then determining their activity following a 50–100-fold dilution in an EGTA-containing assay medium [17]. Such an approach produced the data of Fig. 8C which again document the difference between spinach and pokeweed membranes with respect to their high-affinity interactions with Ca^{2+}. Interestingly, the presence of EGTA in the diluted assay medium of the *Phytolacca* membranes was without consequence for the measured activities. This result suggested that the chelator did not remove any of the activating Ca^{2+} that would not dissociate anyway.

Discussion

It is shown in this article that ever smaller concentrations of added Cl^- satisfied the requirement of the water-oxidizing complex for this anion as the pH was lowered to 5. This was true regardless of the presence of the two extrinsic polypeptides of masses 17 and 23 kDa. However, while the apparent Cl^- affinity increased, the maximal rates of O_2-evolution activity of the polypeptide-depleted membranes membranes declined precipitously below pH 6.3. This activity loss was due to an increased dissociation of activating Ca^{2+}. The observation of a qualitatively opposite pH dependence of the Cl^- and Ca^{2+} association with the water-oxidizing complex was understandable. Cl^- would be expected to interact with positively charged, protonated groups [5] which should be more abundant at low pH, while Ca^{2+} in a biological system typically binds to negatively charged carboxylate groups which proton binding would neutralize [31].

Cl^- probably has both a structural role and a function in the catalytic events of water oxidation. The structural role found its expression in the previously reported stabilizing influence of Cl^- on the association of the water-oxidizing complex with its extrinsic 23 kDa polypeptide [10,11]. The underlying interaction of the anion with this polypeptide presumably accounts for the requirement of at least 6 Cl^- per PS II unit [5,32] and may modulate Cl^- action so as to enable a given amount of bound anion to activate more centers as the pH is lowered.

The Cl^- functioning in the catalytic process of water oxidation assures the orderly progress of charge accumulation in the S-complex of the water oxidase [33–35]. Presumably, it is this Cl^- which is monitored when, as in this investigation, the response of the O_2-evolution activity to the added anion is measured. Thus, the $pK_a < 5$ estimated for the group controlling Cl^- binding in PS II membranes carrying, or depleted of, their 17 and 23 kDa polypeptides, has to be assigned to a group close to the active center, e.g., on the extrinsic 33 kDa, or the intrinsic D_1 and D_2 polypeptides [36]. Lysine, arginine or histidine are likely candidates for Cl^- binding if one invokes groups rendered positively charged by protonation [5,36]. However, of these only histidine might conceivably have a $pK_a < 5$. Alternatively, the low pK_a might reflect a situation similar to that suggested for anion binding to leghemoglobin [37] or for anion adsorption to minerals [38] where putative anion-binding groups are thought to be flanked by negatively charged residues. In the case of Cl^- binding in PS II, such groups might be carboxylates. Their progressive protonation as the pH is lowered would allow Cl^- binding with ever increasing apparent affinity.

The concept of a juxtaposition of carboxylates and positively charged groups like imidazolium

residues could be, with only few additional assumptions, incorporated into the molecular model recently proposed by Coleman and Govindjee [36] for the binding of Cl^- in PS II, and its role in providing a pathway for the protons released during photosynthetic water oxidation [39]. However, it must be stressed that a regulation of the Cl^- affinity by carboxylates would also be compatible with the view of Sandusky and Yocum [40] that Cl^- binding occurs in the Mn-cluster of the water oxidase. The alternative of a competitive interaction between Cl^- and OH^- at this binding site presumably can be ruled out because one would have to invoke actions of nanomolar concentrations of the latter [8].

While for Cl^- both structural and catalytic functions are apparent, the role of Ca^{2+} may be primarily structural [2,5,41] as it is in many other biological systems. A remarkable difference between the responses of PS II membranes to activating Cl^- and Ca^{2+} was that in the case of the former the pH merely changed the apparent K_m, while it created distinct populations of PS II complexes with respect to their ability to be reactivated by added Ca^{2+}. We envisage that the protonation-induced proliferation of low-affinity sites was caused by structural changes of Ca^{2+} binding proteins. Alternatively, the low pH might have favored negative cooperativity of Ca^{2+} binding [27], i.e., the protonated proteins had a tendency to undergo conformational changes in the wake of Ca^{2+} binding with the result that an acquisition of more of the cation became increasingly impeded. Conformational parameters may also explain the resistance of the tightly bound Ca^{2+} to removal by EGTA even though our, and others' [15,17], estimates suggest a dissociation constant of almost 10^{-4} M. We suspect that the reported differences among PS II membranes with respect to their responses to activating Ca^{2+} simply reflect differences in their propensity to undergo conformational changes of the types discussed above.

The well-documented Ca^{2+}-retaining role of the 23 kDa polypeptide can probably be ascribed to a stabilization of the conformation allowing high affinity binding, and to its function as a kinetic barrier to the movement of the cation from and to its binding site (cf. Refs. 12, 18 and 41). The ability to prevent also Cl^- loss may have the same basis [8,24]. However, such functions may have physiological significance only in darkness because our new data suggest that the Cl^- requirement at the slightly acid pH prevailing in the thylakoid lumen during in situ electron transport would be met by the Cl^- content of chloroplasts even in the absence of the two polypeptides. The situation is quite different for Ca^{2+}, however, because in the absence of the 23 kDa polypeptide, the same acid pH would favor the disappearance of high-affinity binding sites. Under those conditions, the Ca^{2+} complement of the water-oxidizing site probably cannot be maintained. Perhaps it is not surprising that the cyanobacterial water-oxidizing complex which lacks analogs of the 17 and 23 kDa polypeptides of chloroplasts [42], has Ca^{2+} requirements quite unlike those found in chloroplast thylakoids [43].

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