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The influence of salts on charge separation ($P680^+ Q_A^-$) and water oxidation of Photosystem II complexes from thermophilic cyanobacteria. Active and inactive conformational states of Photosystem II

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The activities of photosynthetic water oxidation and of the primary stable charge separation have been investigated as a function of the concentration of various salts by measuring flash-induced oxygen yields and absorption changes at 320 nm due to Q_A reduction. (1) At low salt concentrations, water oxidation is inhibited, whereas the charge separation is not affected. (2) The former can be reactivated by salts containing neither calcium nor chloride (e.g., Na_2SO_4), indicating that these ions are not essential cofactors of water oxidation. (3) At higher concentrations, certain salts (e.g., $CaCl_2$) reversibly inhibit water oxidation and in the range of molar concentrations also the primary stable charge separation. (4) These activating and inhibiting effects are explained by equilibria between active and inactive conformational states of PS II. These equilibria depend on the concentrations and properties of the various salts. (5) Arguments are given that, also in higher plants, salt-dependent conformational states are responsible for the salt effects.

Introduction

Photosynthetic water oxidation starts with a light-induced charge separation [1] between a special chlorophyll *a* (P-680, Chl-a_{II}) [2] and a special plastoquinone, Q_A [3], via a pheophytin [4]. The oxidized $P680^+$ is rereduced by a tyrosine [5–7], which in turn consecutively extracts four electrons out of the water-oxidizing complex. The latter, usually designated OEC (oxygen-evolving complex), is thereby oxidized to the states S_1, S_2, S_3 and S_4. S_4 returns spontaneously to the least oxidized state, S_0, together with the release of oxygen due to the oxidation of $2H_2O$ [8,9].

These reactions take place within the protein complex Photosystem II (PS II) formed by at least seven subunits. Two of these are the intrinsic, i.e., membrane spanning, D1 and D2 proteins. They contain the components active in the primary photoreaction [10]. Three extrinsic proteins of 33, 23 and 17 kDa molecular mass play a role in water oxidation [11,12]. The latter two are, however, not present in cyanobacterial PS II [13,14].

Essential for photosynthetic water oxidation are also inorganic cofactors. The most prominent of these is manganese, which stores the redox equivalents within the OEC (for review see Ref. 15). Also calcium and chloride are considered to be essential cofactors that can only be substituted by very similar ions like Sr^{2+} as cation and Br^- and NO_3^- as anions (for review see Refs. 16–18). This role of Ca^{2+} and Cl^- is indicated by results obtained with PS II from higher plants. There, the requirement for these ions has been found to be strongly influenced by the extrinsic 17 and 23 kDa proteins [19]. Usually the calcium requirement can be observed only in the absence of these proteins.

We have avoided this interference by using isolated PS II complexes from a thermophilic cyanobacterium, *Synechococcus* sp., which lacks these two extrinsic pro-

Abbreviations: Chl, chlorophyll; β-DM, dodecyl β-D-maltoside; D1, D2, intrinsic reaction centre proteins of PS II; GdmCl, guanidinium chloride; HMCM, 20 mM Hepes-Na (0.3 M mannitol 20 mM $CaCl_2$/10 mM $MgCl_2$; MMCM, as HMCM, only 20 mM Mes instead of Hepes; OEC, oxygen-evolving complex; P680, primary donor of PS II; Q_A, primary stable quinone acceptor of PS II; SB 12, sulfobetaine 12.

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teins. A different cation requirement compared to higher plants has already been observed with other species of cyanobacteria [20–22]. Furthermore, we studied the effect of salts on water oxidation by measuring flash-induced oxygen yields with a ZrO_2-oxygen sensor [23]. This method is much less susceptible to effects on the acceptor side of PS II than measuring rates of oxygen evolution. It is well known that salts also play a role in the functioning of the acceptor side [24]. We therefore additionally checked the functional integrity of the acceptor side by measuring the amounts of photoreducible Q_A. A preliminary report on some of the following results has been presented at the VIIIth International Congress on Photosynthesis in Stockholm (1989) [25].

Materials and Methods

O_2-evolving complexes from the cyanobacterium *Synechococcus* sp. have been prepared according to Refs. 26 and 27. They were stored at −80°C in 20 mM Mes-Na (pH 6.5), 20 mM $CaCl_2$, 10 mM $MgCl_2$, approx. 1 M sucrose and 0–0.06% SB12. Oxygen yield was measured as described in Ref. 23 with 2 mM $K_3[Fe(CN)_6]$ and 0.2 mM phenyl-*p*-benzoquinone as acceptors ([Chl] ≈ 10–30 μM). The activity of untreated samples was 2.1 to 3.1 mmol O_2 per mol Chl per flash.

Photoreducible Q_A was determined from the initial amplitude of the flash induced absorption change at 320 nm with 2 mM $K_3[Fe(CN)_6]$ and 0.2 mM 2,5-dichloro-*p*-benzoquinone as acceptors ([Chl] ≈ 40–70 μM). Manganese was determined by atomic absorption spectrophotometry as will be described elsewhere [59].

Cl^- and Ca^{2+} concentrations have been determined with ion-selective electrodes having a detection limit of 10 μM Cl^- and 5 μM Ca^{2+}. Determinations were made by way of multiple standard additions using the method according to Gran [28]. Readings of the EMF were taken for the sample itself and after each addition of standard solution. Using a calibration curve, these EMF values were converted to activities, which were plotted against the concentration of added standard, yielding a straight line. The original concentration in the sample is then given by the negative intercept with the abscissa. This method allows determinations of ions practically independent of the sample matrix. The level of contaminations of the stock solutions (buffers and salts) by Ca^{2+} and Cl^- was found to be below the detection limit.

Salt exchange was performed by gel filtration using PD-10 columns by Pharmacia with Sephadex G 25 M. These columns, with an upper exclusion limit of 5 kDa, are very efficient in removing small molecules and ions, reducing their concentrations by a factor of at least 500 [29].

Results

Reactivation of water oxidation by $CaCl_2$ after salt depletion

To remove the salts contained in the storage buffer, the PS II complexes were transferred into other buffer media by gel-filtration, thereby reducing the $CaCl_2$ concentration to less than 25 μM. If no additional salts were present, oxygen evolution was inhibited by more than 80% (Fig. 1). The inhibition was reversed by adding 20 mM $CaCl_2$.

The inhibition becomes irreversible with extended incubation time in the absence of sufficient salt. The irreversible loss of oxygen yield is paralleled by a loss of manganese, while the yield of photoreducible Q_A is practically unaffected. As an example, Fig. 2 shows the relative yields of oxygen and Q_A^- as well as the relative amount of manganese bound to PS II as a function of the incubation time in the presence of 10 mM NaCl at pH 7.5. Similar results were obtained at pH 6.5 and different salt concentrations (5 mM NaCl, 20 mM Na_2SO_4, 40 mM Na_2HPO_4/NaH_2PO_4, not shown). The irreversible inactivation is accelerated by reducing the salt concentration. In the presence of 20 mM $CaCl_2$/10 mM $MgCl_2$, no loss of oxygen yield occurs for at least 10 h at room temperature [29].

In order to distinguish between reversible inhibition and irreversible inactivation by the low salt treatment, the dependence of O_2 yield on the concentration of $CaCl_2$ has been investigated as follows. Immediately after gel-filtration the amount of $CaCl_2$ indicated in Fig. 3 was added to the eluate. One part of it was prepared for oxygen measurement and illuminated about 25 min after gel-filtration by 40 flashes (open circles in Fig. 3, top). To the other part (control sample) a saturating amount of $CaCl_2$ (20 mM) was added

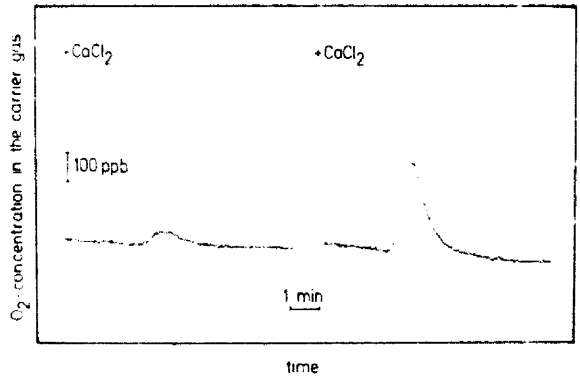

Fig. 1 O_2 yield induced by 40 flashes spaced 1 s apart, with (1.984 nmol O_2) and without (0.327 nmol O_2) readdition of 20 mM $CaCl_2$ after gel filtration (50 mM Mes-Na (pH 6.5)/0.3 M mannitol) ([Chl) = 19 μM, sample volume 1 ml).

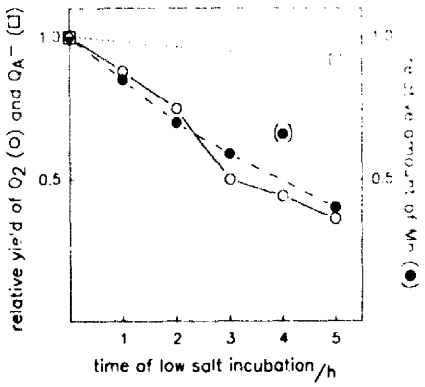

Fig. 2. Relative yield of flash-induced O_2 and Q_A^- formation and the relative amount of Mn bound to PS II as a function of the incubation time in the presence of 10 mM NaCl/20 mM Hepes-Na (pH 7.5)/0.3 M mannitol. The samples were transferred into this medium by gel filtration. 20 mM $CaCl_2$/10 mM $MgCl_2$ were added after the indicated incubation times to stop the deactivation of oxygen evolution.

at the time of illumination of the first part. To correct for irreversible effects the oxygen yield was normalized to the subsequently measured yield of the control sample (closed circles in Fig. 3, top). The normalized data are presented in Fig. 3, bottom. Half-maximal activity is reached at about 130 μM $CaCl_2$.

Reactivation of water oxidation by other salts

The above results are in good agreement with those obtained with PS II from higher plants. However, other salts containing neither calcium nor chloride have been found to reactivate oxygen evolution, too. As an example, Fig. 4 shows the dependence of oxygen yield on the concentration of Na_2SO_4. The data were obtained

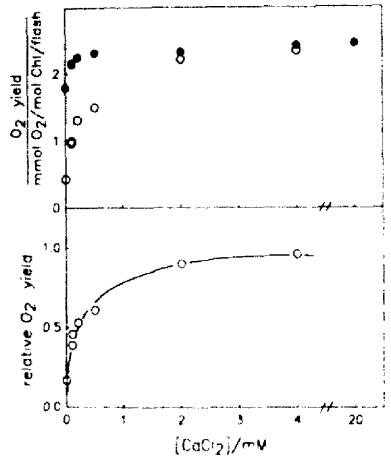

Fig. 3. Top. O_2 yield per Chl and per flash after gel filtration as a function of the concentration of added $CaCl_2$ (open circles) and for control samples (closed circles) as described in the text. Bottom: O_2 yield relative to the control. The gel-filtration column was equilibrated with 50 mM Mes-Na (pH 6.5) and 0.3 M mannitol. The O_2 yield was measured after excitation by 40 flashes spaced 1 s apart.

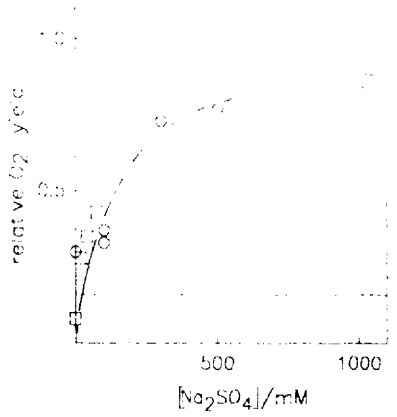

Fig. 4. O_2 yield relative to the control as a function of Na_2SO_4 concentration (squares with 3 mM EDTA, circles without EDTA). Na_2SO_4 and EDTA were added after gel-filtration (squares: 20 mM Mes-Na (pH 6.5)/20 mM Na_2SO_4/0.3 M mannitol; circles: 50 mM Mes-Na (pH 6.5)/0.3 M mannitol). The O_2 yield was measured after excitation by 64 (squares) or 40 flashes (circles), respectively, spaced 1 s apart. The dashed line is an estimate for the relative oxygen yield with 25 μM $CaCl_2$, determined from the upper limit of chloride contamination (50 μM, see text).

in the same way as described for $CaCl_2$. The concentration of Na_2SO_4 needed for half-maximal reactivation is 100–150 mM, i.e., three orders of magnitude higher than that for $CaCl_2$.

To check that the observed effect was not attributable to residual $CaCl_2$ we performed the following experiments:

(a) For depletion of Ca^{2+} that might not have been removed, 3 mM EDTA were added after gel filtration. Reactivation by Na_2SO_4 was not influenced by this addition (squares in Fig. 4). The Ca-EDTA complex has an effective stability constant of $10^{6.7}$ at pH 6.5 and therefore the level of free calcium was far below the detection limit of the Ca^{2+}-selective electrode (5 μM).

(b) The level of contaminating chloride was determined to be less than 50 μM. The dashed line (Fig. 4) shows, as an upper limit of the oxygen yield due to contaminations, the yield that can be estimated for 25 μM $CaCl_2$ from the concentration of half maximal activity in Fig. 3 (130 μM).

(c) We have carried out a treatment with sulphate at elevated pH. This has been described as an optimal method for chloride depletion [30,31]. In the presence of 500 mM Na_2SO_4 PS II complexes were brought to pH 8 by gel-filtration, incubated for 5 min and then brought back to pH 6.5 by a further gel filtration. The O_2 yield then measured was 80% of the yield of an untreated sample.

The effect of various other salts on the oxygen yield is presented in Table I. In these experiments, the PS II complexes were transferred directly into a medium containing the indicated salts, i.e., the column was equilibrated with these media. Otherwise, the controls

TABLE I

Oxygen yield - activation by various salts

Relative O_2 yield corrected (100% = control, see text) and uncorrected (100% = untreated sample) for irreversible inactivation in the presence of the indicated concentrations of various salts. 20 mM Mes-Na (pH 6.5) and 0.3 M mannitol.

Salt	Relative oxygen yield (%)	
	corrected	uncorrected
20 mM $CaCl_2$	100	95
20 mM $MgCl_2$	96	91
60 mM NaCl	100	75
100 mM $MgSO_4$	72	55
500 mM NaH_2PO_4/Na_2HPO_4 (without Mes)	100	99
500 mM Na_2SO_4	96	83
500 mM Mes-Na	68	42

were done as described above. High yields were obtained relative to the control, even in the sole presence of Mes-Na. Although irreversible inactivation appears to be somewhat variable with the different salts (right column of Table I), it is still remarkable that high values of the relative oxygen yield were found at such high salt concentrations.

Reversible inhibition of water oxidation and charge separation by high concentrations of certain salts

It has been reported that, for example, 1 M $CaCl_2$, 1 M $MgCl_2$ and 1 M NaCl inhibit oxygen evolution together with the release of extrinsic proteins [32,11]. We therefore investigated the effect of high concentrations of various salts on the oxygen yield. The results are shown in Table II. It can be seen that $CaCl_2$ and $MgCl_2$ indeed have an inhibitory effect at high concentrations, in contrast to, for example, $MgSO_4$, Na_2SO_4 or NaCl. The absence of an inhibition by NaCl may be related to the absence of the extrinsic 23 and 17 kDa

TABLE II

Oxygen yield inhibition by certain salts

O_2 yield relative to the yield of an untreated sample at 20 mM $CaCl_2$ in the presence of high concentrations of various salts. Besides the indicated salts, the samples contained 20 mM Mes-Na (pH 6.5), 3 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 0.3 M mannitol. Except for $Mg(ClO_4)_2$ and NaSCN, the ionic strength was always 3.3 M.

Salt	Oxygen yield relative to untreated sample at 20 mM $CaCl_2$ (%)
0.60 M $Mg(ClO_4)_2$	5
1.20 M NaSCN	2
1.10 M $CaCl_2$	4
1.10 M $MgCl_2$	30
3.30 M NaCl	86
0.83 M $MgSO_4$	99
1.10 M Na_2SO_4	100

TABLE III

Yield of photoreducible Q_A - inhibition by certain salts

Yield of photoreducible Q_A in the presence of high concentrations of various salts, relative to the yield of an untreated sample at 10 mM $CaCl_2/5$ mM $MgCl_2$. Besides the indicated salts, the samples contained 10 mM Mes-Na (pH 6.5), 10 mM $CaCl_2$, 5 mM $MgCl_2$ and about 0.5 M sucrose.

Salt	Yield of Q_A^- relative to untreated sample at 10 mM $CaCl_2/5$ mM $MgCl_2$ (%)
2.0 M $Mg(ClO_4)_2$	< 5
3.0 M NaSCN	24
3.0 M $CaCl_2$	0
2.0 M $MgCl_2$	25
3.0 M GdmCl	24
3.0 M LiCl	39
3.0 M NaCl	93
1.0 M $MgSO_4$	93

proteins, which are specifically removed from higher plant PS II by 1 M NaCl. With the exception of $Mg(ClO_4)_2$ and NaSCN, all salts were used at the same ionic strength (3.3 M). The inhibitory effect therefore depends on the kind of salt and not just on the charge of the ions. It is furthermore related to the cation (compare, for instance, $CaCl_2$, $MgCl_2$, NaCl) as well as to the anion ($MgCl_2$ vs. $MgSO_4$).

As shown in Table III, at even higher concentrations also the yield of photoreducible Q_A can be completely inhibited. This was observed regardless of the presence of exogenous donors like Mn^{2+}. The efficiency of the various salts in preventing the primary stable charge separation corresponds to the efficiency in the inhibition of oxygen evolution.

Both inhibitory effects are reversible if the excess salt is removed in the dark (see below). This implies that the inhibition occurs already in the dark. While this cannot be shown by a measurement of oxygen yield, because more than one flash is required for O_2 to be evolved, this is possible for the stable charge separation (Fig. 5).

Fig. 5, top left, shows the absorption change at 320 nm due to Q_A reduction upon ten repetitive flashes for an untreated sample. On the right side the average of 256 repetitive flashes is depicted. In the presence of 3 M $CaCl_2$, no absorption change can be observed even upon the first flash (Fig. 5, bottom left). Therefore, the inhibition by $CaCl_2$ occurs already in the dark.

The dependence of O_2 and Q_A^- flash yields on the concentration of $CaCl_2$ is presented in Fig. 6. Oxygen evolution is more susceptible to $CaCl_2$ inhibition than the primary stable charge separation. The reversibility of the inhibition is demonstrated by the open symbols in Fig. 6. These show the relative yields of O_2 and Q_A^- after removal of $CaCl_2$ (at the indicated concentration) in the dark.

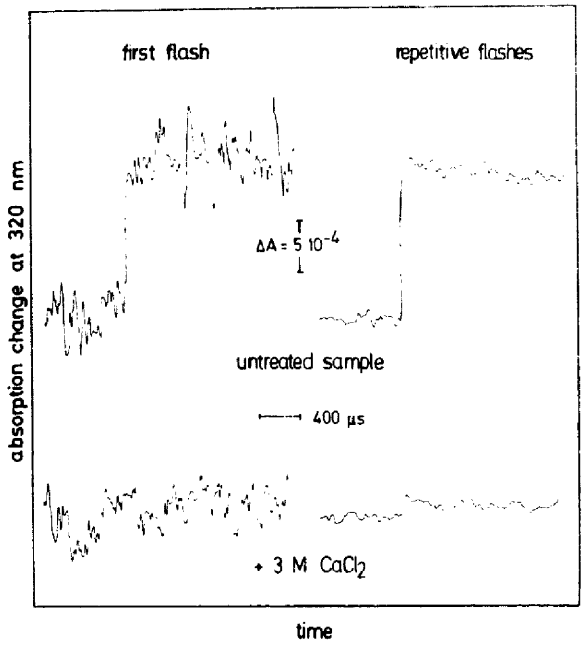

Fig. 5. Absorption changes at 320 nm due to Q_A photoreduction of an untreated sample (top left: average of 10 flashes; top right: average of 256 repetitive flashes) and in the presence of 3 M $CaCl_2$ (bottom left: average of ten first flashes, each flash given to a new dark-adapted sample; bottom right: average of 256 flashes). The samples contained 70 µM Chl, 10 mM Mes-Na (pH 6.5), about 0.5 M sucrose and 0.04% β-DM.

The reversibility of the inhibition of oxygen evolution by $CaCl_2$ is further supported by the following experiment. The oxygen yield induced by eight flashes was measured in the presence of excess $CaCl_2$. After the measurement the sample was passed through a gel-filtration column equilibrated with MMCM (pH 6.5) and again assayed for oxygen yield. While the inhibition was practically complete (rel. O_2 yield < 4%)

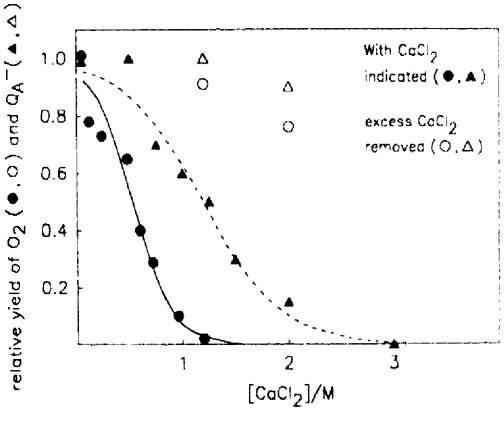

Fig. 6. Flash yields of O_2 and Q_A^- relative to the yield at 20 mM $CaCl_2$/10 mM $MgCl_2$ as a function of the $CaCl_2$ concentration (closed symbols) and after removal of the indicated concentration of excess $CaCl_2$ by gel filtration (open symbols), measured in the presence of 20 mM Mes-Na (pH 6.5)/0.3 M mannitol.

in the presence of 1 M $CaCl_2$, it was 44% reversible even after illumination.

Discussion

Properties of the various salts

As can be seen from the salt concentrations in Table I, the efficiency of the various salts in activating oxygen evolution can be roughly ranked as follows:

$$CaCl_2 \approx MgCl_2 > NaCl > MgSO_4 > Na_2HPO_4 \approx Na_2SO_4 > \text{Mes-Na}$$

A similar order is observed for the inhibition of oxygen evolution and also for the inhibition of charge separation at molar salt concentrations (Tables II and III). This indicates that all three of the observed salt effects may be explained by protein–salt interactions, as the above order of salts is well known to be likewise followed when the influence of salts on protein solubility and on conformation changes of proteins are studied [33].

Based on their effect on protein solubility, the salts are either described as being 'salting out' (decreasing solubility, e.g., Na_2SO_4) or 'salting in' (increasing solubility, e.g., $CaCl_2$) [33,34]. In the order of salts shown above, the 'salting out' salts are found at the right and the 'salting in' salts accordingly on the left.

The solubility of a protein depends on a complex interplay of interactions within the protein, protein–water, protein–salt, but also salt–water interactions. The same interactions determine the conformation of a protein [35]. Thus, a protein will fold in a way to avoid contact of its hydrophobic domains with water. 'Salting out' salts, which are excluded from the protein surface [34], increase the tension at the protein/water interface and therefore promote folding of the protein into native or active conformations [34]. This effect becomes noticeable at concentrations above 100 mM. The unfavourable protein–water interaction can also be minimized by precipitation of the protein.

'Salting in' salts tend to bind to the protein [36]. Binding of salts to localized sites on the protein surface stabilizes active conformations, an effect which usually saturates at a tenth millimolar salt [35]. At higher concentrations the solubility of proteins is increased by unspecific binding of salt (e.g., to the peptide bonds [33]), but also unfolded, inactive protein conformations are favoured. In the case of protein complexes subunit dissociation may occur [33]. The binding of 'salting in' salts outweighs the effect of surface tension increase [34].

Model for salt induced conformational changes between active and inactive states

Based on these considerations we propose that the activating and inhibiting salt effects on the oxygen

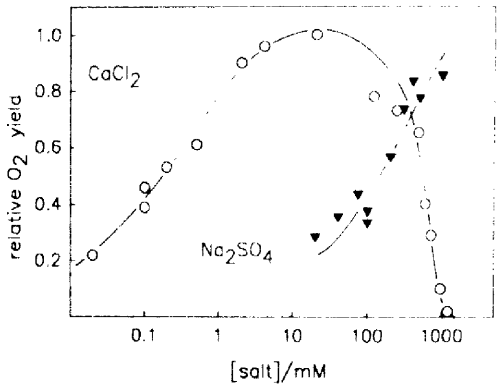

Fig. 7. The relative O_2 yield as a function of the $CaCl_2$ (circles) and the Na_2SO_4-concentration (triangles) on a logarithmic scale (data of Figs. 3, 4 and 5).

yield, which are summarized in Fig. 7, are explained by a salt-dependent shift of equilibria between an active (A) and two inactive conformational states of PS II (I_1 and I_2). At molar salt concentrations, a further, state (I_3) exists which is not capable of forming the $P680^+Q_A^-$ pair. We are aware that, in view of the complexity of PS II, there is probably more than one active conformation and also many different conformations that are reversibly inactivated. Still, our simple model can give at least a qualitative explanation of our results. The proposed states of our model can be characterized as follows.

In the absence of sufficient salt, the equilibrium is shifted towards the inactive state, I_1. Manganese may slowly dissociate from this conformational state as it is not properly stabilized by its proteinaceous ligands. This explains why the reversibility of the inhibition decreases with longer incubation times. It may also indicate that the folding of the protein in this inactive state is less tight than in the active state.

Either by binding of 'salting in' salts (concentration range 0.05–5 mM) or by surface tension increase due to the 'salting out' salts (50–1000 mM), the equilibrium is shifted towards the active state A. The different mechanisms of stabilization of the active state may explain the drastically different values for the concentration at half-maximal activation (130 μM $CaCl_2$, 100–150 mM Na_2SO_4 (Fig. 7)).

Increased binding of 'salting in' salts (100–1000 mM) to PS II shifts the equilibrium beyond the optimum to the second inactive state, I_2. This state may be due to at least partial unfolding of the PS II proteins and a reversible dissociation of the 33 kDa protein. Manganese, however, is not lost [32,59]. As the intramembrane parts of PS II may be removed from contact with water or salts through detergent molecules, the unfolding may occur only in the extrinsic proteins or in those loops of the intrinsic proteins that protrude into the aqueous phase.

That the inhibition of oxygen evolution by high concentrations of $CaCl_2$ is due to an unfolding is supported by our observation that the primary stable charge separation, $P680^+Q_A^-$, is also reversibly inhibited by molar concentrations (i.e., > 0.5 M) of 'salting in' salts. As the dissociation of the D1/D2 heterodimer or a reversible extraction of Q_A by these salts are very unlikely, an unfolding transition to a further state I_3 is the only reasonable explanation. As can be expected for such a transition, it occurs already in the dark (Fig. 5).

The activation of oxygen evolution by $CaCl_2$ (Fig. 3) follows a saturation curve, i.e., it can be explained by binding of $CaCl_2$ to the inactive state I_1:

$$I_1 + CaCl_2 \rightleftharpoons A$$

The concentration dependence for the inhibition of oxygen evolution and charge separation by $CaCl_2$ at high concentrations (Fig. 6) has a shape different from that of the curve of activation. The former are similar to the reversible denaturation observed with other proteins [37] in respect to the high concentration range and the sigmoidal shape. Such curves can be described by a linear concentration dependence of the standard free enthalpy change (ΔG^0) for the transition from a native (active) to an unfolded (inactive) state, i.e., for water oxidation:

$$A \rightleftharpoons I_2$$

or for charge separation:

$$A' \rightleftharpoons I_3$$

The linear dependence can be rationalized by considering the excess free enthalpy due to the thermodynamically nonideal behaviour of the conformational states [38], which can be expected at such high salt concentrations. Interestingly, the reactivation of water oxidation by a 'salting out' salt (Na_2SO_4, Fig. 7),

$$I_1 \rightleftharpoons A$$

occurs in the same range of high concentration as the inhibition by $CaCl_2$, a typical 'salting in' salt, and might be described in a similar way.

The above discussion can be briefly summarized as follows:

(a) The various salt effects are explicable by salt-dependent conformational changes in the protein complex of PS II.

(b) In the different conformational states, water oxida-

tion and charge separation are either active or reversibly inhibited.
(c) That the concentration range for the activation of water oxidation can differ by up to three orders of magnitude (e.g., $CaCl_2$ vs. Na_2SO_4) can be explained by the different properties of the salts (salting in, salting out).

Relevance of the proposed model for PS II from higher plants

Different from our results for the activation of oxygen evolution by various salts (Table I), a pronounced specificity for calcium and chloride has been observed with PS II from higher plants (for review see Refs. 16–18). There, Ca^{2+} was found to be replaceable only by Sr^{2+} [39,40], while chloride can be substituted by similar monovalent ions like Br^- and NO_3^- [41]. We observed that Ca^{2+} could be effectively replaced by Mg^{2+} or Na^+ and Cl^- by SO_4^{2-}, HPO_4^{2-} or even the Mes anion. In contrast to this, there are reports on an inhibitory effect of sulphate for PS II from higher plants [42,31,43], although this was not observed by other authors [30,44]. Based on the observed specificity, it was proposed that Ca^{2+} and Cl^- are part of the manganese cluster in the OEC [30,45].

For the case of calcium, the distinct stoichiometry found per PS II may support such a specific role of this ion. In PS II from higher plants, two Ca^{2+} per Q_A have been determined [46,47]. In a rice mutant capable of oxygen evolution [48] as well as in PS II preparations from *Synechococcus* [49] only one Ca^{2+} is found per Q_A. It is still a matter of controversy whether special treatments that inhibit oxygen evolution in higher plant PS II either reversibly (1 M NaCl, citrate (pH 3)) or irreversibly (Tris, NH_2OH) are accompanied by or even due to a release of the tightly bound calcium ions [46,47,50,51]. Therefore, it is not clear whether these are involved in the process of water oxidation at all.

Even for higher plants, EPR and EXAFS experiments have so far been unsuccessful in demonstrating direct chloride binding to manganese in the OEC. In Ref. 44 an anion-dependent interchange between the EPR multiline signal and the $g = 4.1$ signal was observed, however. A similar effect, on the other hand, can also be induced by certain cryoprotectants (glycerol, ethyleneglycol) or ethanol [52]. Therefore, these effects are probably due to conformational changes in the protein, as the latter substances have been shown to interact with proteins in a way similar to the 'salting out' salts (see Ref. 34), but are not likely to bind to the manganese in the OEC directly [53].

It appears possible that specifically bound Ca^{2+} and Cl^- ions have not been removed in our experiments. However, the treatments for salt depletion used in this work (dilution (either by washing or gel filtration), EDTA, pH 8 + Na_2SO_4) are in principle the same as those used by other workers in this field. Furthermore, we observed not only the same effect of salt depletion, i.e., reversible inhibition of oxygen evolution, but also a similar concentration dependence for the reactivation by $CaCl_2$. Therefore, it is reasonable to assume that specifically bound $CaCl_2$ was removed to the same extent as in the experiments of other authors.

In this context it is of note that we observed a slow irreversible loss of oxygen yield even in the presence of 10 mM Cl^- (see Fig. 2). Therefore, this irreversible inactivation cannot be explained by a slow release of chloride. That also a loss of manganese occurs under this condition indicates that chloride is not specifically involved in stabilizing the manganese.

The question has to be answered as to how the different reactivating effects of salts in higher-plant and cyanobacterial PS II can be explained. One possible answer is that in the course of evolution, a specific requirement of water oxidation for calcium and chloride may have developed. Then these ions must be part of the mechanism of water oxidation in higher plants, but not in cyanobacteria. Two different mechanisms are not likely, however, because the structure of the manganese complex in the OEC is very similar for both types of organism, as far as it can be investigated by EXAFS and EPR measurements [54,55]. At least the D1, D2 [56] and the extrinsic 33 kDa protein [14] are also very similar.

Therefore we believe that in higher plants the mechanism of water oxidation is in principle the same as in cyanobacteria. Accordingly, the observed salt effects in both cases may be explained by salt induced changes between active and inactive conformational states of the PS II-protein complex. However, the dependence of these equilibria on the various salts is different in higher plants, very probably because the PS II-complex is different, at least in respect to the extrinsic 17 and 23 kDa proteins. This argument (see also Ref. 57) is supported by the fact that in higher plants the requirement for Ca^{2+} and Cl^- is strongly influenced by these extrinsic proteins. Furthermore, the degree of Ca^{2+}-depletion that can be achieved depends on these extrinsic proteins. It has been shown that a complete removal (down to less than 1 Ca^{2+} per PS II) requires washing with 2 M NaCl [58]. Without this treatment, extensive washing of PS II core preparations does not yield less than 2 Ca^{2+}/PS II, but reduces oxygen evolution almost completely (25% of the control).

The reversible inhibition of the primary stable charge separation by molar concentrations of 'salting out' salts was first observed here with cyanobacterial PS II. Because this effect can be explained on the basis of general protein–salt interactions, it is likely that it is also observable in PS II from higher plants, in bacterial reaction centres and possibly also in Photosystem I. As the corresponding conformational changes are ex-

pected to be very drastic, it should be worthwhile to investigate these by methods yielding more direct structural information.

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