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THE EFFECTS OF MONO- AND DIVALENT SALTS ON THE O_2-EVOLUTION ACTIVITY AND LOW TEMPERATURE MULTILINE EPR SPECTRUM OF PHOTOSYSTEM II PREPARATIONS FROM SPINACH

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The ability of salts to inhibit the O_2-evolution activity of PS II preparations is shown to parallel closely the Hofmeister series, suggesting that inhibition is related to the solubility of the 16, 24 and 33 kDa proteins in these salt solutions. An examination of the effect of salt inactivation on the low temperature multiline EPR signal indicates that the release of either the 16 and 24 kDa proteins, or additionally the 33 kDa protein blocks or greatly reduces the efficiency of the advancement of the water-splitting complex to the S_2-state; under some conditions, this inhibition is reversible.

The recent development of highly active O_2-evolving Photosystem II (PS II) preparations [1–3] has provided the foundation for a more thorough understanding of the water-splitting apparatus in green plants. A number of groups have shown that treatment of PS II preparations with high concentrations of NaCl results in the partial loss of O_2-evolution activity and the release of a 24 kDa protein and a 16 kDa protein accompanied by a negligible loss of manganese [4–6]. Reconstitution studies indicate that the 24 kDa protein can rebind and substantially restore the O_2 evolution activity of deactivated samples [5–8], but conflicting results have been published concerning the necessity of rebinding the 16 kDa protein for activity [7,8]. Recently, Ono and Inoue [9] have shown that 1 M $CaCl_2$ or $MgCl_2$ produces nearly complete inactivation of O_2-evolution and additionally extracts the 33 kDa protein without releasing manganese. The rebinding of the 33 kDa protein to $CaCl_2$-inactivated preparations restores about 30% of the original activity [10], suggesting that this protein is also essential for competent water-splitting activity.

In this report, we examine the effects of mono- and divalent salts on the O_2-evolution activity and amplitude of the low-temperature multiline EPR signal [11,12] of PS II preparations and find that (1) the ability of salts to inhibit oxygen evolution closely follows the Hofmeister series, and (2) inactivation by salts or elevated pH is accompanied by the loss of multiline EPR signal associated with the S_2-state; under some conditions, the inhibition is reversible.

All preparative procedures were carried out at 0 or 4°C. Chloroplast thylakoid membranes were prepared as in (1). O_2-evolving PS II preparations were obtained by the procedure described by Kuwabara and Murata (2), with the following exceptions: (1) the Triton X-100 extraction was accomplished in a medium containing 15 mM

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Abbreviations: Chl, chlorophyll; PS II, Photosystem II; Mes, 4-morpholineethanesulfonic acid.

NaCl/5 mM $MgCl_2$/50 mM Mes at pH 6.0, (2) a buffer containing 50 mM Mes/10 mM NaCl at pH 6.0 (hereafter designated the 'control buffer') was used in the two subsequent wash cycles.

The procedures for salt deactivation and reactivation by dialysis are described in the legends. Steady-state measurements of O_2 evolution were made polarographically, using Yellow Springs Instrument 4004 electrode and a high sensitivity teflon membrane. The continuous illumination protocol used to generate the low-temperature multiline EPR signal has been published [12]; data collection and analysis were performed as described previously [12]. The peptide content of the extracts and pellets was analyzed by SDS polyacrylamide gel electrophoresis according to the method of Laemmli [13], and compared to those obtained after treatment with 960 mM Tris (pH 9.3), a condition known to release completely all three peptides [4]. Chlorophyll concentrations were obtained by the method of Arnon [14]. Manganese was analyzed using a Perkin-Elmer 360 flame atomic absorption spectrophotometer.

The dependence of O_2 evolution inactivation at pH 6.0 on the concentration of sodium salts, Na_2SO_4, NaCl and NaBr, is shown in Fig. 1A, while that for the magnesium salts, $MgSO_4$ and $MgCl_2$, is given in Fig. 1B. Treatment with $CaCl_2$ resulted in an inhibition curve similar to that obtained for $MgCl_2$. All of the salts tested inactivated O_2 evolution to some degree, but their effectiveness as inhibitors differed significantly (Fig. 1). This variability could not be correlated with the ionic strength of the medium. For example, 800 mM Na_2SO_4 or $MgCl_2$ yield solutions of equal ionic strength, yet the preparation treated with the Na_2SO_4 retained 15- to 20-fold more activity (Fig. 1). At 800 mM salt, inhibition incresed in the order $Na_2SO_4 < MgSO_4 \approx NaCl < NaBr < MgCl_2 \approx CaCl_2$.

Several groups have shown that PS II preparations inactivated with NaCl [4,5] or $CaCl_2$ and $MgCl_2$ [9] lose very little manganese. We have also observed that after a 2 h incubation in the control buffer plus either 800 mM Na_2SO_4, NaCl, NaBr, $MgSO_4$, $MgCl_2$, or $CaCl_2$, the manganese content of the extracted preparations (pellet fraction) remains within 10% of the control level (4.6 Mn/250 Chl). Thus, the loss of activity cannot be ascribed to a loss of essential manganese for any of these salt treatments.

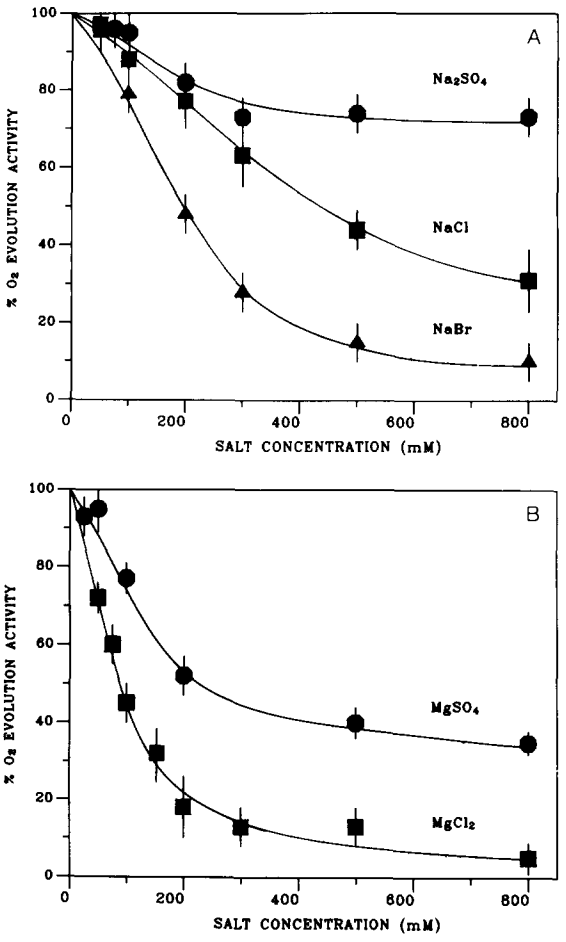

Fig. 1. The effect of salt concentration on the steady-state rates of O_2 evolution from the PS II preparation at pH 6.0 (A) Na_2SO_4 (●); NaCl, (■); NaBr, (▲). (B) $MgSO_4$, (●); $MgCl_2$, (■). Samples were suspended to a concentration of 1–3 mg Chl/ml in 50 mM Mes/10 mM NaCl (pH 6.0) buffer (control buffer) plus the indicated concentrations of salt at 0°C and incubated for 2 h under room light at this temperature. The membranes were then pelleted (34000 × *g*, 10 min) and resuspended in the control buffer. Oxygen evolution was assayed at 21 ± 2°C in the control buffer, which additionally contained ferrocyanide and ferricyanide, each 3 mM, and 500 μM 2,5-dichlorobenzoquinone as an electron acceptor system. Error bars indicate ± 1 standard deviation about the average of at least three determinations. Control rates of O_2-evolution activity usually obtained were between 300 and 400 μmol O_2 per mg Chl per h, with rates as high as 500 μmol O_2 per mg Chl per h occasionally observed.

Fig. 1 reveals that the ability of salts to act as inhibitors depends on both the cation and anion of

the salt. For a given cation inhibition increased in the series of anions $SO_4^{2-} < Cl^- < Br^-$, while for a given anion inhibition increased in the series of cations $Na^+ < Mg^{2+} \approx Ca^{2+}$. This sequence of inhibition shows a close correspondence to the Hofmeister or lyotropic series and suggests that the inactivation of O_2-evolution depends on the relative solubilities of the 16, 24 and 33 kDa proteins in these salts. Thus, NaCl, a monovalent salt that acts as a weak 'salting-in' agent [15,16] produces about 70% inactivation at 800 mM (Fig. 1) and extracts only the 16 and 24 kDa proteins [4–7], while $MgCl_2$ and $CaCl_2$, divalent salts that act as strong 'salting-in' agents [15,16], produce close to complete inactivation at 0.8–1 M (Fig. 1, Ref. 9) and additionally extract the 33 kDa protein [9]. For comparison, we find that the divalent salt, $MgSO_4$, produces about 60% inactivation at 800 mM (Fig. 1) and releases only the 24 and 16 kDa proteins, while the monovalent salt, NaBr, causes about 90% inactivation at 800 mM (Fig. 1) and also partially extracts the 33 kDa protein. This reveals that the inhibition of O_2-evolution and extraction of the 16, 24 and 33 kDa proteins is not strictly related to the valency of the salt.

Recently, a multiline EPR signal has been observed at low temperature [11], and evidence has accumulated indicating that the signal is associated with manganese in the S_2-state of the O_2-evolving site [12,17,18]. Incubation of PS II preparations in high pH buffers, which are known to cause the release of manganese [4], produced a sharp decrease in the amplitude of the multiline signal between pH 8.0 and 9.0, along with a loss of O_2 evolution activity (data not shown). In contrast salt-treated preparations lose very little manganese, but both $MgCl_2$- and NaCl-treated samples also showed a decrease in the amplitude of the multiline signal that paralleled the loss of O_2-evolution (Table I).

The O_2-evolution activity and EPR multiline spectrum amplitude of salt-treated samples which retained above about 10% of the control activity could both be substantially restored by simply dialyzing the high-salt membranes against the control buffer for 12–14 h, using low molecular weight

TABLE I

THE EFFECT OF SALT DEACTIVATION AND REACTIVATION BY DIALYSIS ON THE AMPLITUDE OF THE LOW-TEMPERATURE MULTILINE EPR SIGNAL

Samples were suspended to a concentration of 1–3 mg Chl/ml for 2 h in the control buffer plus the indicated concentrations of salt, and were then assayed for O_2-evolution activity and multiline signal amplitude as described in Fig. 1 and in the text. To reactivate, aliquots of the high-salt preparations were dialyzed against the control buffer for 12–14 h using 3500 MW cut-off tubing (Spectrapor). The dialyzed samples were pelleted, resuspended in the control buffer and then assayed for O_2-evolution activity and multiline signal amplitude. The O_2-evolution activities and amplitudes of the multiline spectrum are normalized to that of the control samples.

Salt (mM)	Deactivation		Reactivation	
	Activity [a] (% of control)	Multiline amplitude [b] (% of control)	Activity [c] (% of control)	Multiline amplitude [b] (% of control)
NaCl				
200	84	88	82	94
500	47	53	75	72
800	22	50	74	83
$MgCl_2$				
50	68	69	86	81
100	40	55	79	96
200	12	41	77	63
300	9	16	62	62
500	7	23	45	28
800	5	15	21	6

[a] Control activities: NaCl, 412 μmol O_2 per mg Chl per h; $MgCl_2$, 399 μM O_2 per mg Chl per h.

[b] Estimated uncertainty in the relative amplitudes of the multiline signal, approx. 10%.

[c] Control activities after dialysis: NaCl, 400 μmol O_2 per mg Chl per h; $MgCl_2$, 309 μmol O_2 per mg Chl per h.

cut-off tubing (Table I). For example, samples suspended in 300 mM $MgCl_2$ exhibited only about 9% of the control O_2 evolution rate and 16% of the control multiline spectrum amplitude, but after dialysis 62% of the control O_2-evolution activity and multiline spectrum amplitude were observed. However, the ability to restore activity and multiline spectrum amplitude depended on the original degree of inactivation; after suspension in 800 mM $MgCl_2$, the level of O_2-evolution activity after dialysis rose to only 20% of the control value, and the amplitude of the multiline signal did not increase significantly (Table I). No restoration of O_2-evolution was obtained when only the pellets of the salt-treated samples were dialyzed against the control buffer, clearly indicating the necessity of higher-molecular-weight components in the supernatants for water-splitting activity.

Previous salt inactivation [4–6] and reconstitution studies [5–8,10] have shown that the 33 kDa, the 24 kDa and possibly the 16 kDa proteins are necessary for competent O_2-evolution activity. Suspension of PS II preparations in NaCl (4–6) or low concentrations of $MgCl_2$ [10] releases only the 16 and 24 kDa proteins, while high concentrations of $MgCl_2$ additionally release the 33 kDa protein [9,10]. Our results more specifically indicate that the release of either the 16 and 24 kDa proteins by NaCl or the additional release of the 33 kDa protein by $MgCl_2$ disrupts the electron-transport chain in a fashion that blocks or greatly decreases the efficiency of the advancement of the water-splitting complex to the S_2-state. We have found that EPR signal II_f is produced during continuous illumination of salt-extracted PS II preparations (Boska, M., Blough, N.V. and Sauer, K., unpublished data), which indicates that the site of inhibition is prior to signal II. These results support the recent work of Åkerlund et al. [19], which suggested that salt-washing affects S-state turnover.

Under conditions in which the 16 and 24 kDa proteins are released, the ability to restore a significant portion of the O_2-evolution activity and multiline signal amplitude after removal of salt by dialysis (Table I) suggests that these proteins do rebind to reestablish functional electron-transport and water-splitting activity, as reported previously [5–8]. However, under conditions in which the 33 kDa protein is also substantially released (800 mM $MgCl_2$) our results indicate that inhibition is not largely reversible (Table I). This suggests that the 16, 24 and 33 kDa proteins are not properly rebinding to establish a competent water-splitting complex. This inability to reactivate substantially after release of the 33 kDa protein may be due to the partial loss of manganese [19] or Ca^{2+} [20–22] during the dialysis procedure.

In conclusion, although manganese is not released concurrently with the salt-extraction of the 16, 24 and 33 kDa proteins from PS II preparations at pH 6.0, the formation of the low-temperature multiline EPR signal attributed to manganese in the S_2-state of O_2-evolving complex is blocked under conditions in which these proteins are released. The EPR signal and the O_2-evolution activity are significantly restored after the dialysis of PS II preparations suspended in salts that release primarily the 16 and 24 kDa proteins.

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