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Protection of the oxygen-evolving Photosystem II complex by glycinebetaine

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The effect of glycinebetaine, a nontoxic osmolite of halophilic plants, on the photosynthetic oxygen evolution was investigated with Photosystem II (PS II) particles prepared from spinach thylakoids and another type of PS II particles (dPS II particles) that had been depleted of the 18 kDa and the 23 kDa extrinsic proteins. Betaine protected PS II particles from the inactivation of oxygen evolution by high concentrations of NaCl, in which the chaotropic effect of Cl^- dissociated the 18 kDa and 23 kDa extrinsic proteins from the oxygen-evolving PS II complex. However, it failed to protect dPS II particles, in which the inactivation of oxygen evolution was caused by the effect of Na^+ ions.

Introduction

Halophilic plants such as Chenopods synthesize glycinebetaine (hereafter, betaine) as an osmoprotective substance in the cytoplasm and in the chloroplast [1]. Its concentration in spinach chloroplasts reaches 0.3 M when the plant is grown in saline environments [2]. In addition to its osmoregulatory role, betaine has been shown to stabilize complex enzymes, such as ribulose-1,5-bisphosphate carboxylase/oxygenase [3,4], phospho*enol*pyruvate carboxylase [5], pyruvate kinase [6] and glucose-6-phosphate dehydrogenase [7], in high-salt media. It is likely that betaine prevents the dissociation of these complex enzymes into subunits at high concentrations of NaCl [3,7].

The oxygen-evolving PS II complex in the chloroplasts is composed of more than 10 intrinsic membrane proteins and three extrinsic proteins of 18, 23 and 33 kDa [8,9]. When PS II particles containing the PS II complex are exposed to high concentrations of NaCl, the 18 kDa and 23 kDa proteins dissociate but the 33 kDa protein remains attached. This treatment leads to the inactivation of oxygen evolution [10,11].

In the present work, we investigated the effect of betaine on the salt-induced inactivation of the photosynthetic oxygen evolution in two types of Photosystem II complex: the oxygen-evolving PS II complex with all three extrinsic proteins embedded in PS II particles [9], and the oxygen-evolving PS II complex with the same components but lacking the 18 kDa and 23 kDa extrinsic proteins embedded in dPS II particles [10]. Our results show that betaine protects the oxygen-evolving complex in PS II particles from the dissociation of extrinsic proteins by salt and the concomitant inactivation of oxygen evolution. However, it fails to protect the complex in the dPS II particles from the inactivation of oxygen evolution by salt.

Materials and Methods

PS II particles having a Chl *a*: Chl *b* ratio of 1.96 : 1 were isolated by treating spinach thylakoids with Triton X-100 according to Kuwabara and Murata [12]. They were suspended in 0.025 M Mes-NaOH (pH 6.5)/0.30 M sucrose/0.01 M NaCl, at 2.5–2.7 mg Chl ml^{-1} and were stored in liquid nitrogen.

To prepare dPS II particles, the PS II particles were suspended in 1.2 M NaCl/0.30 M sucrose/0.025 M

Abbreviations: Chl, chlorophyll; dPS II particles, Photosystem II particles depleted of the 18 kDa and the 23 kDa extrinsic proteins; Mes, 4-morpholineethanesulfonic acid; PBQ, phenyl-*p*-benzoquinone; PS II, Photosystem II; SDS, sodium dodecylsulfate.

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Mes-NaOH (pH 6.5) and were incubated for 30 min at 0 °C. Subsequently, they were washed by centrifugation at 33 000 × *g* for 10 min and resuspension first in 0.20 M NaCl/0.30 M sucrose/0.025 M Mes-NaOH (pH 6.5) and then in 0.01 M NaCl/0.30 M sucrose/0.025 M Mes-NaOH (pH 6.5). Finally they were suspended in 0.01 M NaCl/0.50 M sucrose/0.025 M Mes-NaOH (pH 6.5) at 3 mg Chl ml^{-1}, and were stored in liquid nitrogen.

For the assays of oxygen evolution, the frozen suspension was thawed and the particles were collected by centrifugation at 33 000 × *g* for 10 min and resuspended at 1.3–1.5 mg Chl ml^{-1} in either of the following two media for the assay: 0.025 M Mes-NaOH (pH 6.5)/0.30 M sucrose (hereafter Mes-sucrose); and 0.025 M Mes-NaOH (pH 6.5)/1.2 M betaine (hereafter Mes-betaine). The Cl^- concentration, determined with a Cl^--specific electrode (Toko Chemical Laboratories, Tokyo, Japan), was 0.126 mM in both media. Data cited in this work are not corrected for the background concentration of Cl^- in the media. Supplementary additions of salts to the reaction mixtures are given in Results.

Photosynthetic oxygen evolution was measured with a Clark-type oxygen electrode [12]. The reaction mixtures containing 6–8 μg Chl ml^{-1}, 0.3 mM PBQ, plus additions mentioned in individual experiments, were made in the Mes-sucrose or the Mes-betaine medium. Samples were incubated for 3 min before turning on the actinic light. Illuminations lasted 1 min. Chl was determined according to Arnon [13].

Dissociation of the 18, 23 and 33 kDa extrinsic proteins from PS II particles after various salt treatments was estimated by analyzing a fraction of each protein remaining attached to PS II particles. The frozen suspension of PS II particles were thawed and collected by centrifugation as mentioned above. PS II particles were incubated for 10 min at 0 °C in a designated medium, and then they were collected by centrifugation at 33 000 × *g* for 30 min and subjected to analysis of polypeptide composition by SDS-gel electrophoresis (polyacrylamide concentration, 5% in stacking gel and 14% in separation gel). Gels contained 6.0 M urea in the buffer system of Laemmli [14]. The gel was stained with Coomassie brilliant blue R-250 and the electrophoretic pattern was recorded at 560 nm with a TLC scanner (CS930, Shimadzu, Kyoto, Japan). The amount of each protein was determined according to peak height of the stained band in the densitogram, with the untreated PS II particles serving as a control.

Results

Tolerance of PS II and dPS II particles against betaine

First, the question was posed as to whether PS II and dPS II particles tolerated betaine, because some of the reaction mixtures contained high concentrations of betaine. Fig. 1 shows the effects of betaine and NaCl on oxygen evolution by PS II and dPS II particles. Both types of particle tolerated high concentrations of betaine, but were inactivated by high concentrations of NaCl. Oxygen evolution of the dPS II particles was depressed in the absence of NaCl (Fig. 1B). This effect is likely to reflect the Cl^- requirement for oxygen evolution in the oxygen-evolving complex depleted of the 18 kDa and 23 kDa proteins [15].

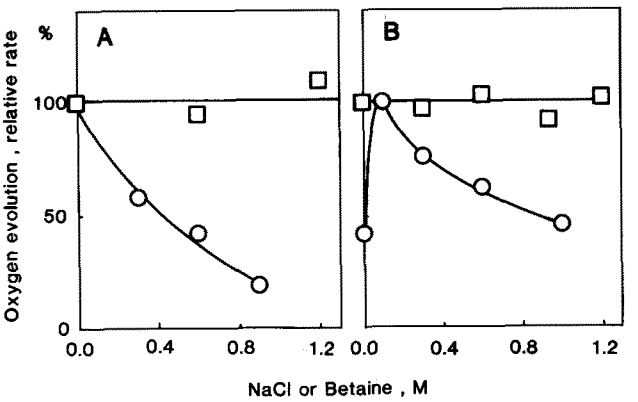

Fig. 1. Effects of various concentrations of NaCl and betaine on oxygen evolution. (A) PS II particles. (B) dPS II particles, been depleted of the 18 kDa and 23 kDa extrinsic proteins. Reaction mixtures were made in 0.025 M Mes-NaOH, 0.001 M $CaCl_2$, 0.30 M sucrose (pH 6.5). □, Betaine; ○, NaCl. In the case of the betaine concentration curves the reaction mixtures were supplemented with 0.024 M NaCl. Oxygen-evolving activities corresponding to relative rates of 100 were 500 μmol O_2/mg Chl per h for PS II particles and 310 μmol O_2/mg Chl per h for dPS II particles.

Effects of betaine on PS II particles

Fig. 2 shows the effects of betaine on the NaCl-induced inactivation of oxygen evolution by PS II par-

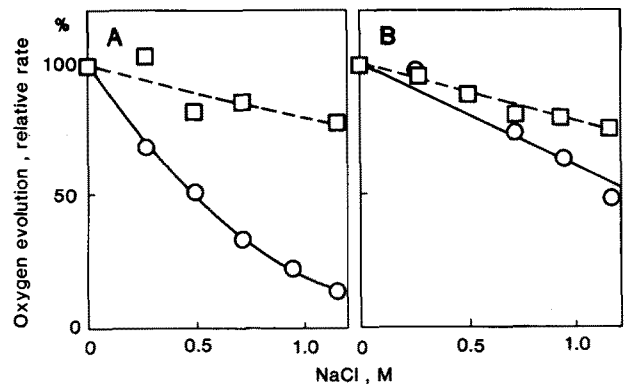

Fig. 2. Effects of various concentrations of NaCl on oxygen evolution of PS II particles suspended either in (A) 0.025 M Mes-NaOH (pH 6.5)/0.30 M sucrose (Mes-sucrose), or in (B) 0.025 M Mes-NaOH (pH 6.5)/1.2 M betaine (Mes-betaine). ○———○, no $CaCl_2$ added; □— — —□, 0.01 M $CaCl_2$ added. Oxygen-evolving activities (μmol O_2/mg chl per h) corresponding to relative rates of 100 were 320 for PS II particles in Mes-sucrose, 390 for the same plus 0.01 M $CaCl_2$, 310 for PS II particles in Mes-betaine, and 397 for the same plus 0.01 M $CaCl_2$.

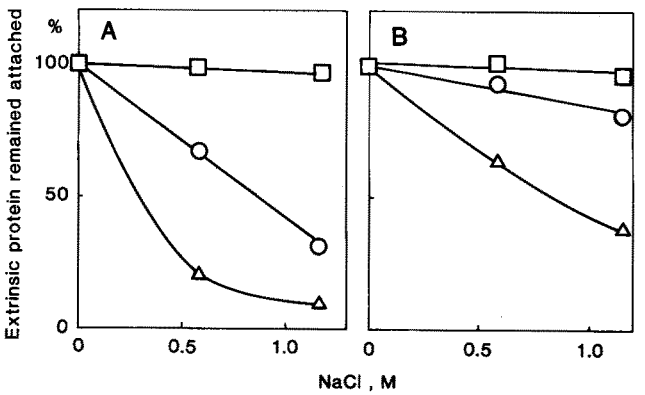

Fig. 3. Effects of NaCl on the dissociation of the extrinsic proteins from PS II particles. PS II particles were incubated for 10 min at 0 °C with the indicated concentration of NaCl either in (A) 0.025 M Mes-NaOH (pH 6.5)/0.30 M sucrose (Mes-sucrose), or in (B) 0.025 M Mes-NaOH (pH 6.5)/1.2 M betaine (Mes-betaine). The incubated PS II particles were collected by centrifugation, and the pelleted PS II particles were subjected to electrophoretic analysis, as described in Materials and Methods. △——△, 18 kDa protein; ○——○, 23 kDa protein; □——□, 33 kDa protein.

ticles. In Mes-sucrose (Fig. 2A), the oxygen-evolving activity of PS II particles measured in 0.5 M NaCl was 50% of the control level measured in the absence of added NaCl, and that in 1.0 M NaCl was only 20% of the control level. In the presence of 1.2 M betaine, i.e., in Mes-betaine (Fig. 2B), on the other hand, the oxygen-evolving activities in 0.5 M and 1.0 M NaCl were 80% and 60%, respectively, of the control level. These findings demonstrate that the high concentration of betaine protects the oxygen-evolving PS II complex from inactivation by high concentrations of NaCl. When the same experiment was carried out in Mes-sucrose and the Mes-betaine media supplemented with 0.01 M $CaCl_2$, the inactivations in 0.5 M and 1.0 M NaCl were only 10% and 20%, respectively (Fig. 2A), and there was no protective effect of betaine (Fig. 2B). These results may be explained by the previous findings that the high concentrations of NaCl dissociate the 23 kDa extrinsic protein from the oxygen-evolving complex and that the exogenously added Ca^{2+} ions could substitute for the functions of this extrinsic protein [16–18]. The same type of experiment was carried out with choline-Cl in place of NaCl. Essentially the same results as in Fig. 2 were obtained in the inactivation of oxygen evolution and the protective effect of betaine.

Fig. 3 shows the effects of betaine on the NaCl-induced dissociation of the 18, 23 and 33 kDa extrinsic proteins from the oxygen-evolving complex. In Mes-sucrose (Fig. 3A), the 18 kDa and 23 kDa proteins, but not the 33 kDa protein, were dissociated from the complex at high concentrations of NaCl; the dissociation of the 18 kDa protein was 2 or 3-times as extensive as that of the 23 kDa protein, as reported previously [10]. In the presence of 1.2 M betaine, i.e., in Mes-betaine, the binding of these proteins was stabilized; the extents of dissociation of the 18 kDa and the 23 kDa proteins were 60% and 20%, respectively, in the presence of betaine, as compared with 100% and 70%, respectively, in the absence of betaine. The same type of experiment was carried out with choline-Cl in place of NaCl. Although choline-Cl was less effective than NaCl in the dissociation of the extrinsic proteins, essentially the same result as in Fig. 3 was obtained. This result suggests that the Cl^- anions but not the Na^+ cations are effective in the dissociation of the extrinsic proteins and on the inactivation of oxygen evolution.

To investigate the effect of other anions on the oxygen evolution by PS II particles, PS II particles were suspended in 0.4 M of several sodium salts and oxygen-evolving activity was measured (Table I). Among Na_2SO_4, NaCl, NaBr and $NaNO_3$, Na_2SO_4 was the least effective; 90% of the control activity remained. NaBr was more effective than NaCl. The greatest effect in the inactivation of oxygen evolution was observed with $NaNO_3$, which completely eliminated the oxygen-evolving activity. Betaine and $CaCl_2$ protected the oxygen evolution from the inactivation by these salts. The inactivating effects of the tested salts increased in the order of $Na_2SO_4 < NaCl < NaBr < NaNO_3$, irrespective of the presence or absence of betaine and $CaCl_2$. This order is in parallel with the ranking order

TABLE I

Effects of sodium salts on the oxygen-evolving activity of PS II particles

The PS II particles were incubated for 10 min at 0 °C in 0.40 M of the indicated salt either in 0.025 M Mes-NaOH (pH 6.5)/0.30 M sucrose (Mes-sucrose) or in 0.025 M Mes-NaOH (pH 6.5)/1.2 M betaine (Mes-betaine) in the presence or absence of 0.006 M $CaCl_2$.

Salt added during incubation (0.40 M)	Oxygen evolution (μmol O_2/mg Chl per h; (% of control))			
	in Mes-sucrose		in Mes-betaine	
	$-CaCl_2$	$+CaCl_2$	$-CaCl_2$	$+CaCl_2$
None (control)	384 (100)	403 (100)	440 (100)	462 (100)
Na_2SO_4	324 (89)	359 (89)	431 (98)	462 (100)
NaCl	157 (41)	305 (76)	313 (71)	421 (91)
NaBr	59 (15)	212 (53)	236 (54)	298 (65)
$NaNO_3$	0 (0)	0 (0)	62 (14)	123 (27)

TABLE II

Effects of sodium salts on the dissociation of the extrinsic proteins from PS II particles

The PS II particles were incubated for 10 min at 0 °C with 0.40 M of the indicated salt either in 0.025 M Mes-NaOH (pH 6.5)/0.30 M sucrose (Mes-sucrose) or in 0.025 M Mes-NaOH (pH 6.5)/1.2 M betaine (Mes-betaine). The incubated PS II particles were collected by centrifugation and the pelleted PS II particles were subjected to electrophoretic analysis, as described in Materials and Methods.

Salt added during incubation (0.40 M)	Fractions of proteins remained attached to PS II particles (% of control)					
	in Mes-sucrose			in Mes-betaine		
	18 kDa	23 kDa	33 kDa	18 kDa	23 kDa	33 kDa
None (control)	100	100	100	100	100	100
Na_2SO_4	54	90	100	81	98	100
NaCl	21	66	99	83	100	100
NaBr	4	32	100	43	81	98
$NaNO_3$	4	17	99	19	62	95

of anions in the Hofmeister series of chaotropicity [19,20].

Table II shows the effect of various salts on the dissociation of the extrinsic proteins from PS II particles. In Mes-sucrose, the 18 kDa protein was almost completely dissociated by $NaNO_3$ and NaBr, but less by NaCl and Na_2SO_4. The dissociation of 23 kDa protein was much less than that of 18 kDa protein with all the salts, and that of 33 kDa protein was negligible. When the extents of dissociations of the extrinsic proteins were compared, the effectiveness was in the order of $Na_2SO_4 < NaCl < NaBr < NaNO_3$. This order is the same as that observed in the inactivation of oxygen evolution. Betaine had a protective effect on the binding of these extrinsic proteins, but did not change the order of effectiveness of salts.

Effects of betaine on dPS II particles

Fig. 4 shows the effects of high concentrations of NaCl and choline-Cl on the oxygen-evolving activity of

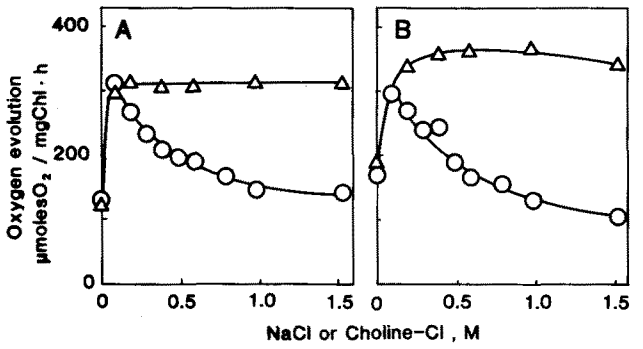

Fig. 4. Effects of NaCl and choline-Cl on oxygen-evolving activity of dPS II particles. ○———○, NaCl; △———△, choline-Cl. Reaction mixtures were made either in (A) 0.025 M Mes-NaOH (pH 6.5)/0.30 M sucrose, or in (B) 0.025 Mes-NaOH (pH 6.5)/1.2 M betaine, and were supplemented with 0.001 M $CaCl_2$.

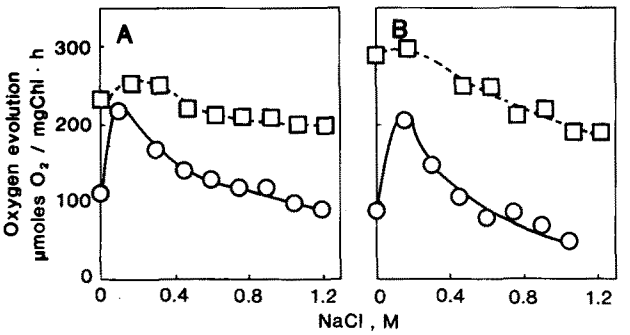

Fig. 5. Effects of NaCl on oxygen-evolving activity of dPS II particles. The particles were suspended either in (A) 0.025 M Mes-NaOH (pH 6.5)/0.30 M sucrose (Mes-sucrose) or in (B) 0.025 M Mes-NaOH (pH 6.5)/1.2 M betaine (Mes-betaine). ○———○, 0.001 M $CaCl_2$; □— — —□, 0.01 M $CaCl_2$.

dPS II particles. Below 0.1 M of NaCl and choline-Cl, the photosynthetic oxygen evolution was depressed, as observed in Fig. 1B. Above 0.1 M, however, they produced different responses. The dPS II particles became progressively inactivated with increasing concentration of NaCl, while no inactivation was observed at high concentrations of choline-Cl. This observation suggests that the NaCl-induced inactivation of oxygen evolution by dPS II particles is due to Na^+. Betaine did not protect the oxygen-evolving complex in dPS II particles against the NaCl-induced inactivation (Fig. 4B).

Fig. 5 shows the effects of Ca^{2+} ions on the inactivation of oxygen evolution by NaCl in the presence or absence of betaine. The inactivation by NaCl was much suppressed in the medium containing 0.01 M $CaCl_2$ compared with the medium containing 0.001 M $CaCl_2$. Nearly identical responses were obtained with particles suspended in Mes-sucrose (Fig. 5A) and in Mes-betaine (Fig. 5B). This suggests that there is no interaction between the effect of Ca^{2+} and that of betaine in dPS II particles.

TABLE III

Effects of sodium salts on the oxygen-evolving activity of dPS II particles

The dPS II particles were incubated for 10 min at 0 °C in 0.40 M of the indicated salt either in 0.025 M Mes-NaOH (pH 6.5)/0.30 M sucrose (Mes-sucrose) plus 0.001 M $CaCl_2$, or in 0.025 M Mes-NaOH (pH 6.5)/1.2 M betaine (Mes-betaine) plus 0.001 M $CaCl_2$.

Salt added during incubation (0.40 M)	Oxygen evolution (μmol O_2/mg Chl per h; (% of control))	
	in Mes-sucrose	in Mes-betaine
None (control)	198 (100)	272 (100)
Na_2SO_4	85 (43)	104 (38)
NaCl	69 (35)	110 (40)
NaBr	27 (17)	69 (25)
$NaNO_3$	39 (20)	41 (15)

Table III presents the effect of 0.4 M of several sodium salts on the oxygen evolution by dPS II particles. The extent of inactivation of oxygen evolution ranged from about 80% in the case of NaBr and $NaNO_3$ to about 60% in the case of NaCl and Na_2SO_4. This indicates that the effectiveness of salts was rather constant irrespective of various anions. Although the rate of oxygen evolution by dPS II particles suspended in Mes-betaine was higher than that in Mes-sucrose, the inactivation presented in the relative rate (the values in parentheses of Table III) was independent of the presence of betaine.

Discussion

Betaine is an osmoprotective substance and is physiologically important to halophilic plants [21]. It is water-soluble, non-toxic, suitable for maintaining the osmotic balance of the cell. It is also effective in protecting the structure of complex enzymes from dissociation by NaCl [3,7]. However, it has been uncertain whether it is capable of protecting the oxygen-evolving Photosystem II complex. In this paper, we addressed this question. The results suggest that betaine protects the oxygen-evolving PS II complex from the dissociation caused by NaCl.

Effects of betaine on PS II particles

High concentrations of NaCl dissociated the 18 kDa and the 23 kDa extrinsic proteins from the oxygen-evolving PS II complex and suppressed its oxygen-evolving activity [10,18]. Choline-Cl had essentially the same effects as NaCl on the dissociation of extrinsic proteins and the suppression of oxygen evolution. We observed that the ability of the salts to dissociate the extrinsic proteins followed the anion's position in the Hofmeister series of chaotropicity [20]; this is consistent with the findings reported by Blough and Sauer [22]. These observations suggest that the inactivation of oxygen evolution and the dissociation of extrinsic proteins are caused by the chaotropic effect of Cl^-. The counter cation, Na^+, may not play an essential role in this effect.

Betaine protected the oxygen-evolving complex from the dissociation of the extrinsic proteins and the inactivation of oxygen evolution. The parallelism between the two types of protection in the PS II particles suggests that the main effect of betaine is to stabilize the binding of the extrinsic proteins to the intrinsic part of the oxygen-evolving PS II complex. As a result, oxygen evolution is protected by betaine, since the 23 kDa extrinsic protein is necessary for oxygen evolution if a sufficient concentration of Ca^{2+} is not supplemented [8,17]. This is consistent with the observation that, in the presence of 0.01 M Ca^{2+} which can substitute for the function of the 23 kDa protein, the dissociation by NaCl and the stabilization by betaine of the 23 kDa protein did not affect the oxygen-evolving activity (Fig. 2). It has been demonstrated that the 18 kDa protein is necessary for oxygen evolution when the Cl^- concentration is lower than 0.01 M. Under the conditions of NaCl concentrations employed in the present study, the dissociation of the 18 kDa protein appears to be unrelated to the inactivation of oxygen evolution.

Effects of betaine on dPS II particles

High concentrations of NaCl suppressed oxygen evolution of dPS II particles, but choline-Cl did not show such an effect. These observations suggest that Na^+ cations, but not Cl^- anions, are effective in this inactivation. In the presence of 0.01 M Ca^{2+}, the effect of NaCl was very much suppressed. As suggested by Waggoner et al. [23], Na^+ may displace Ca^{2+} which has been bound to its functional site.

Betaine has essentially no effect on the inactivation of oxygen evolution by NaCl in dPS II particles. This may suggest that betaine cannot stabilize the binding of Ca^{2+} at its functional site.

Conclusions

(1) In PS II particles suspended in high concentrations of NaCl, the chaotropic action of Cl^- anions dissociates the 23 kDa and 18 kDa extrinsic proteins from the oxygen-evolving PS II complex, and as a result suppresses oxygen-evolving activity. Betaine protects the PS II complex from the dissociation of extrinsic proteins by NaCl, and thus sustains oxygen-evolving activity.

(2) In dPS II particles suspended in high concentrations of NaCl, Na^+ ions displace, from their functional site, Ca^{2+} ions which become necessary for oxygen evolution in the PS II complex depleted of the 23 kDa extrinsic protein, and thus inactivate oxygen evolution. Betaine does not prevent the inactivation caused by Na^+ cations.

(3) It is suggested that in halophilic plants living under saline conditions NaCl, intruded into the chloroplasts, causes the same effect as in PS II particles, and that betaine, accumulated in the chloroplasts, protects the oxygen-evolving PS II complex from the dissociation of the extrinsic proteins.

Acknowledgements

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