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Stabilization of the Mn-cluster of the oxygen-evolving complex by glycinebetaine

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The protective effect of glycinebetaine on inactivation of the photosynthetic oxygen evolution was studied in isolated Photosystem II particles from spinach that had been depleted of all three extrinsic proteins of the oxygen-evolving complex (ddPS2 particles). Glycinebetaine protected the ddPS2 particles against the inactivation by incubation at room temperature and at alkaline pH which may perturb the structure of the proteins that coordinate the Mn-cluster. However, glycinebetaine was not effective in protecting the ddPS2 particles against inactivation caused by incubations with NH_2OH and with $Mn(CH_3COO)_2$, that are known to interact directly with the Mn-culster. It did not prevent photoinhibition of ddPS2 particles either.

Introduction

The catalytic site for the photosynthetic evolution of oxygen is a cluster of four manganese ions [1]. It has been suggested that the Mn-cluster is coordinated by ligands of more than 20 acidic amino acids of the intrinsic proteins, D1 and D2, of PS II [2–4]. Glu-69 of D2 protein [3] and Asp-170 of D1 protein [5,6] have been identified as such ligands. The intrinsic protein, CP47, in the PS II core complex may also be involved in the ligation of manganese ions [7,8].

The 33-kDa extrinsic protein associates strongly with the intrinsic proteins, with a dissociation constant of 12 nM, and provides sites for the tight association of the 23-kDa and 18-kDa extrinsic proteins [9]. PS II particles that have been depleted of the three extrinsic proteins (ddPS II particles) release half of their bound manganese ions much more rapidly than do native PS II particles [10]. These results suggest that, after the dissociation of the 33-kDa protein, conformational changes in the Mn-coordinating proteins occur that facilitate the departure of half of the manganese ions from the cluster.

It has been shown that glycinebetaine (hereafter referred to as betaine), a compatible osmolyte, blocks the inactivation of soluble enzymes by high concentrations of NaCl [11]. When some higher plants, such as spinach, are exposed to high-salt conditions, they synthesize betaine in the stroma of chloroplasts [12,13]. Recently, we showed that betaine stabilizes the binding of the extrinsic proteins to the intrinsic domain of PS II complex of spinach [14,15] and, thus, protects the evolution of oxygen against inactivation by high concentrations of salts [15]. A similar type of protective effect of betaine has been observed in the oxygen evolution by intact thylakoid membranes isolated from cyanobacteria [16–18], suggesting that betaine can penetrate into the lumen to stabilize the Mn-cluster through preservation of the attached 33-kDa extrinsic protein.

In the present study we addressed the question of whether or not betaine can stabilize the evolution of oxygen by directly protecting the Mn-cluster, when all the extrinsic proteins are absent. Our results demonstrate that betaine protects the Mn cluster against lytic and alkaline-induced inactivation.

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Abbreviations: Bis-Tris propane, 1,3-bis(trishydroxymethyl)-methylamino propane; Chl, chlorophyll; D1 and D2, intrinsic proteins of the Photosystem II core complex; DCIP, 2,6-dichlorophenol indophenol; ddPS II, Photosystem II particles depleted of the 18-, 23- and 33-kDa extrinsic proteins; DPC, 1,5-diphenyl carbazide; Mes, 4-morpholinoethanesulfonic acid; PBQ, phenyl-*p*-benzoquinone; PS II, Photosystem II particles prepared by extraction with Triton X-100; SDS, sodium dodecyl sulphate; Tyr_z, photo-oxidizable Tyr residue that mediates the transfer of electrons from the Mn-cluster to the primary electron donor of Photosystem II.

Materials and Methods

PS II particles were prepared from spinach thylakoids as described previously [19]. The PS II particles were incubated in 300 mM sucrose, 200 mM NaCl, 10 mM $CaCl_2$, 25 mM Mes-NaOH (pH 6.5) and 3.0 M urea [20] at 0°C for 10 min. The suspension was centrifuged at $35000 \times g$ for 15 min, and the pelleted particles were suspended at a concentration that corresponded to 1.0 mg Chl/ml in 300 mM sucrose, 200 mM NaCl, 10 mM $CaCl_2$ and 25 mM Mes-NaOH (pH 6.5) (hereafter designated medium S). The ddPS II particles suspended in medium S were stored in liquid nitrogen. These particles contained extrinsic proteins at 10–15% of the original levels found in PS II particles, as determined by SDS-PAGE [20].

The rate of oxygen evolution was determined at 25°C by monitoring the change in concentration of oxygen with a Clark-type oxygen electrode (model 5300; Biological Monitor, Yellow Springs, OH, USA) for 30 to 60 s after the start of actinic illumination. Under these conditions, the photoinactivation of oxygen evolution during the assay was practically negligible. Reaction mixtures in medium S contained ddPS II particles that corresponded to 6–8 μg Chl/ml and 0.3 mM PBQ as the exogenous electron acceptor. Each mixture was stirred in the reaction vessel for 3 min in subdued light to bring the temperature to 25°C before exposure to actinic light. Actinic light with wavelengths from 600 to 760 nm and at an intensity of 640 W/m^2 was obtained by passage of light from an incandescent lamp through optical filters (VR60 and HA50; Hoya Glass, Tokyo, Japan).

The reduction of DCIP was measured as described elsewhere [19] with a dual-wavelength spectrophotometer (UV300; Shimadzu, Kyoto, Japan). Wavelengths of measuring and reference beams were 580 nm and 500 nm, respectively, and the slit width was set at $\Delta\lambda = 2$ nm. Reaction mixtures contained ddPS II particles that corresponded to 4 μg Chl/ml, 16 μM DCIP and 500 μM DPC, when present, in medium S. Actinic light with wavelengths from 650 to 760 nm and at an intensity of 800 W/m^2 was provided from an incandescent lamp through optical filters (VR65 and HA50; Hoya Glass, Tokyo, Japan). Changes in concentrations of DCIP were calculated from changes in absorbance for 30 s after the start of illumination. A differential absorption coefficient ($\Delta\epsilon_{580-500} = 10.88$ mM^{-1}cm^{-1}) of DCIP was calculated for the optical parameters of our assay system by use of the absorption coefficient of DCIP at pH 6.5 that was reported by Armstrong [21].

Treatments of ddPS II particles at different pH values were carried out by incubating the particles in 300 mM sucrose, 200 mM NaCl, 10 mM $CaCl_2$ and 40 mM Bis-Tris propane (medium P). For treatments of ddPS II particles with NH_2OH, with $Mn(CH_3COO)_2$ and with light, the particles were incubated in medium S.

The amount of manganese in ddPS II particles was assayed by inducibly coupled plasma emission with an ICP spectrophotometer (SPS1200; Seiko Instruments, Tokyo, Japan). Chl was estimated by the method of Arnon [22].

Results and Discussion

Effects of betaine on ion requirements

It has been shown that ddPS II particles require 200 mM Cl^- ions [20] for the evolution of oxygen. We examined whether betaine modifies the requirement for Cl^- ions of the ddPS II particles for the evolution of oxygen. Fig. 1 shows that the dependence on the concentration of Cl^- ions was the same in the presence of 1.0 M betaine and in its absence, with half-maximal activity appearing at 60 mM choline-Cl and maximal activity at 200 mM choline-Cl. However, maximal activity was enhanced by 40% in the presence of betaine. Essentially the same results were obtained when choline-Cl was replaced by NaCl, suggesting that the requirement for choline-Cl can be attributed to a requirement for Cl^- ions.

The ddPS II particles also require 5–10 mM Ca^{2+} ions if they are to support a significant rate of evolution of oxygen [23]. In the presence of betaine, the requirement for Ca^{2+} ions was saturated at approx. 5 mM (data not shown). Thus, betaine did not alter the requirements for Cl^- and Ca^{2+} ions of the ddPS II particles for the evolution of oxygen.

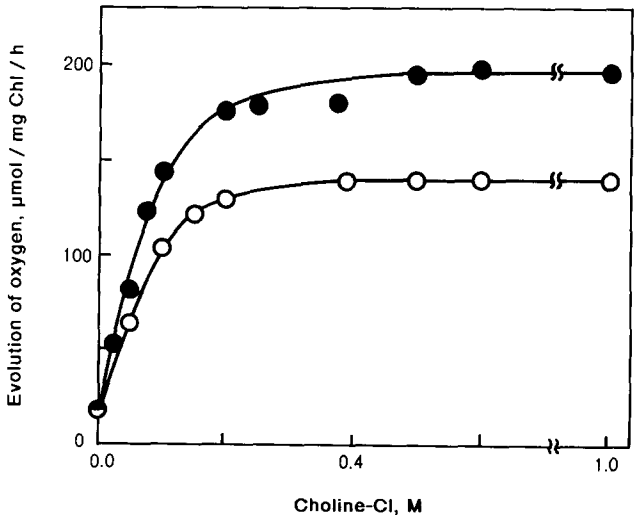

Fig. 1. The effects of betaine on the oxygen-evolving activity of ddPS II particles at various concentrations of choline-Cl. ddPS II particles were suspended at a concentration of Chl that corresponded to 10 μg/ml in 300 mM sucrose, 10 mM $CaCl_2$, 25 mM Mes-NaOH (pH 6.5), and the designated concentrations of choline-Cl. ○———○, no betaine added; ●———●, 1.0 M betaine.

These observations indicate that betaine does not compete with the cation or the anion for their specific binding sites. One possible explanation for the absence of any effect of betaine on the requirements for these ions is that betaine is too large to gain access to these binding sites, which can be accessed only by small ions, such as Br^-, NO_3^- and La^{3+}, which are known to compete with Cl^- and Ca^{2+} ions [24,25].

Inactivation of the evolution of oxygen at room temperature

A time-dependent loss of activity occurs when ddPS II particles are incubated at room temperature in darkness [10,20]. We compared betaine and glycerol, another osmolyte of salt-stressed organisms, for their abilities to protect the oxygen-evolving complex of ddPS II particles during incubation at 25°C in darkness (Fig. 2). When incubated at 25°C in darkness in the suspension medium alone, or in the presence of 1.37 M glycerol, which corresponds to 10% glycerol (v/v), the ddPS II particles lost their oxygen-evolving activity with a half-inactivation time of approx. 80 min. By contrast, the activity barely changed at all during incubation for 3 h in the presence of 1.0 M betaine. Choline-Cl at 1.0 M also preserved the oxygen-evolving activity of the ddPS II particles (data not shown), although choline is not a natural osmolyte. This observation suggests that the protective effect of betaine in ddPS II particles can be attributed to its quaternary amine.

It was recently reported that both betaine and polyhydroxylic solutes protect PS II particles [26] and PS II core particles [27] from thermal inactivation of oxygen evolution. However, our implication is in disagreement with the interpretation of Homann [26] and Williams and Gounaris [27], who proposed that the zwitterionic betaine and the electroneutral polyalcohols and sugars exert similar protective effects.

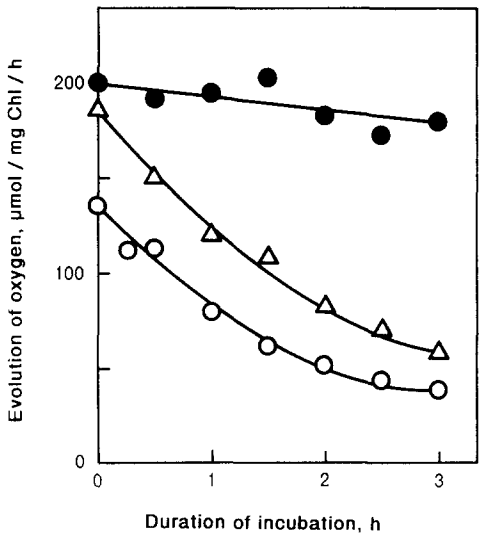

Fig. 2. The effects of betaine and glycerol on the inactivation of the oxygen evolution during incubation of ddPS II particles at 25°C in darkness. ddPS II particles were suspended at a concentration that corresponded to 10 μg Chl/ml in medium S supplemented with glycerol or betaine. After incubation at 25°C for designated periods of time, the oxygen-evolving activity of ddPS II particles in the suspension was assayed directly. ○——○, no glycerol, no betaine; △——△, 1.37 M glycerol; ●——●, 1.0 M betaine.

We compared the inactivation of the oxygen evolution with the release of manganese from the ddPS II particles during incubation at 25°C in darkness in the presence and in the absence of betaine (Fig. 3). In the absence of betaine, the oxygen-evolving activity and the manganese content declined in parallel. In the pres-

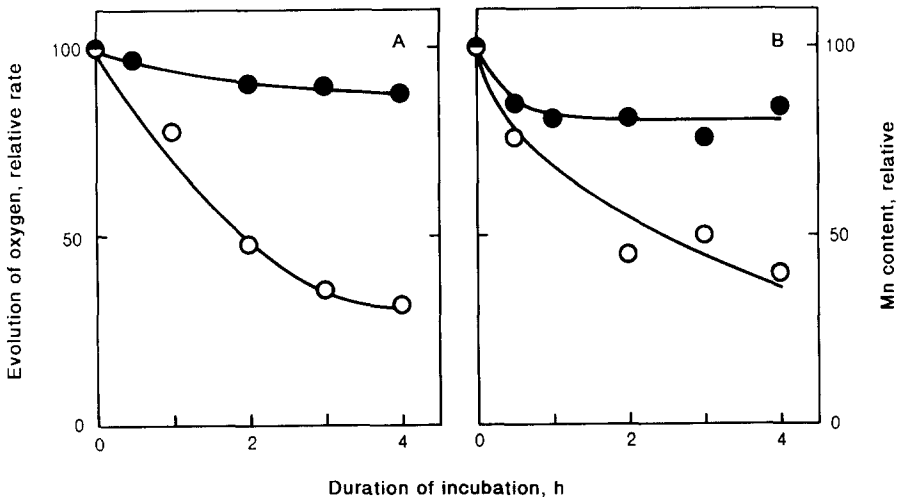

Fig. 3. The effects of betaine on the inactivation of the oxygen evolution and the release of manganese during incubation of ddPS II particles at 25°C in darkness. ddPS II particles were suspended in medium S that contained 1.0 mM EDTA in order to remove traces of unbound and adsorbed Mn^{2+} ions, and they were collected by centrifugation at 20000× *g* for 7 min. Then they were suspended at a concentration that corresponded to 10 μg Chl/ml in medium S with or without 1.0 M betaine. After a defined period of incubation, part of the suspension was withdrawn for an assay of the evolution of oxygen, and the remainder was centrifuged at 20000× *g* for 7 min to pellet the ddPS II particles for the assay of manganese. (A) The oxygen-evolving activity. (B) The Mn content of particles. The relative value of 100 corresponds to 3.8 Mn/200 Chl (mol/mol). ○——○, no betaine added during incubation; ●——●, 1.0 M betaine added during incubation.

ence of betaine, by contrast, the changes in both parameters were small. These observations suggest that betaine stabilizes the evolution of oxygen by preserving the Mn-cluster in ddPS II particles.

Inactivation of the oxygen evolution at alkaline pH

We investigated the effect of betaine on the inactivation of the oxygen evolution of ddPS II particles under alkaline conditions (Fig. 4). The oxygen-evolving activity was not affected by incubation for 20 min at pH 6.5 or at pH 7.0, irrespective of the presence or absence of betaine. However, the activity was almost entirely lost at pH 8.0 in the absence of betaine and at pH 9.0 in the presence of betaine. Half-maximal inactivation was attained at pH 7.5 in the absence of betaine and at pH 8.0 in the presence of betaine.

We further examined the effects of various concentrations of betaine in protecting the ddPS II particles against inactivation at pH 8.0 of the transport of electrons from H_2O to DCIP (Table I). The protective effect of betaine increased with increasing concentrations up to 1.35 M, but no further protection was observed at higher concentrations of betaine, for example, 1.8 M. We also investigated the effect of alkaline pH on the transport of electrons from DPC to DCIP. Neither the alkaline treatment nor the presence of betaine had any effect on this reaction. These observations suggest that betaine protected the oxygen evolution, but not the photochemical reaction of PS II, against inactivation under alkaline conditions.

Inactivation of the oxygen evolution by NH_2OH, $Mn(CH_3COO)_2$ and light

We examined effects of chemicals, such as NH_2OH and Mn^{2+} ions, that are known to affect the Mn-cluster directly [28,29]. The results in Fig. 5 show that betaine did not significantly protect the ddPS II particles from inactivation by NH_2OH and $Mn(CH_3COO)_2$.

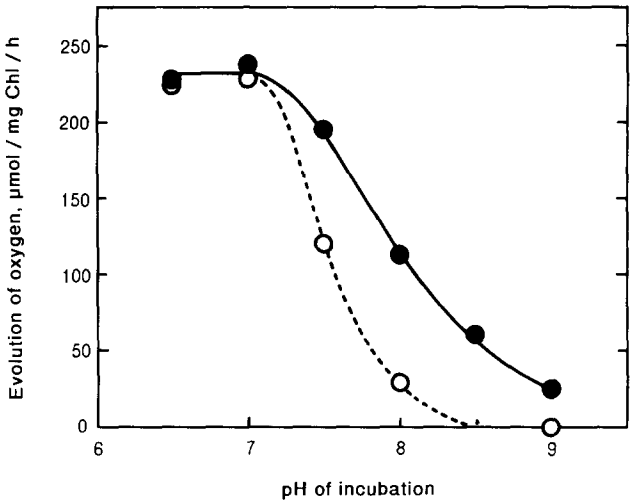

Fig. 4. The effects of betaine on the inactivation of the oxygen evolution of ddPS II particles at alkaline pH. ddPS II particles were suspended, at a concentration that corresponded to 150 μg Chl/ml, in medium P at the designated pH with or without 1.0 M betaine. After incubation for 20 min at 0°C in darkness, the particles were collected by centrifugation at 20000× *g* at 0°C for 7 min. Then they were resuspended in medium S that contained 1.0 M betaine and their oxygen-evolving activity was assayed. ○———○, no betaine added to the incubation medium; ●———●, 1.0 M betaine added to the incubation medium.

It has been suggested that NH_2OH and Mn^{2+} ions act as donors of electrons to PS II and reduces manganese in the cluster [4,30,31]. It has also been suggested that these chemicals interact with the manganese in the cluster and act as ligands at or near the Cl^--binding site [32]. The results in this study suggest

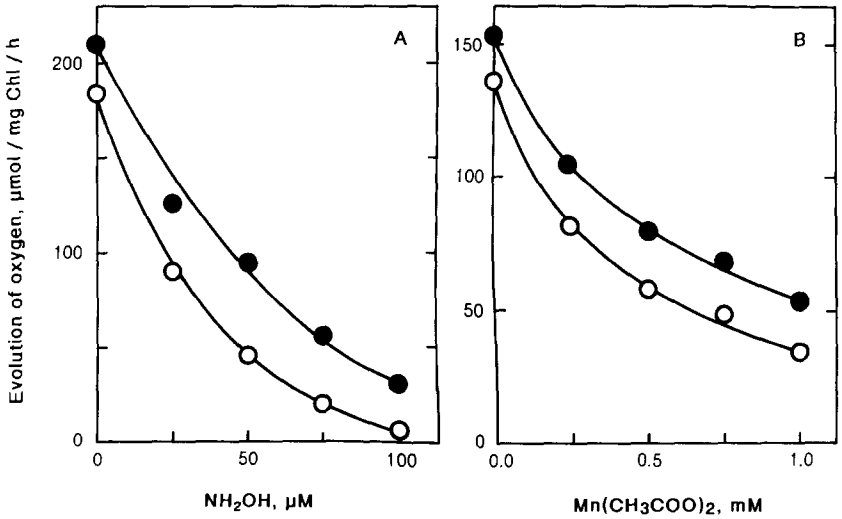

Fig. 5. The effects of betaine on the inactivation of the oxygen-evolving complex of ddPS II particles during incubation of the particles with NH_2OH and $Mn(CH_3COO)_2$. ddPS II particles were suspended at a concentration that corresponded to 150 μg Chl/ml in medium S supplemented with defined concentrations of the compounds in the presence of betaine or in its absence. After incubation at 0°C in darkness for 60 min, the particles were collected by centrifugation at 20000× *g* at 0°C for 7 min. Then they were resuspended in medium S that contained 1.0 M betaine for the assay of oxygen-evolving activity. ○———○, no betaine added to the incubation medium; ●———●, 1.0 M betaine added to the incubation medium.

TABLE I

The effects of betaine on the inactivation of the electron transport of ddPS II particles by incubation at pH 8.0

The ddPS II particles were suspended at a concentration that corresponded to 4 μg Chl/ml in medium P, at pH 8.0, which was supplemented with indicated concentrations of betaine. After incubation for 30 min at 0°C, particles were collected by centrifugation at 0°C at 20000× g for 20 min and suspended in medium S supplemented with 1.0 M betaine. Electron transport was assayed by monitoring reduction of DCIP at 25°C in medium S with 0.1 mM DCIP, or 0.1 mM DCIP plus 0.5 mM DPC. A relative rate of reduction of DCIP of 100 corresponds to reduction of 76 μmol DCIP/mg Chl per h.

Betaine (M)	Electron transport (relative rate)	
	$H_2O \rightarrow$ DCIP	DPC → DCIP
0	19	100
0.45	30	98
0.90	42	99
1.35	74	108
1.80	73	94

that betaine has no effect on the direct interaction of these chemicals with the Mn-cluster in the PS II complex.

Photoinactivation of the oxygen evolution

The photochemical reaction of PS II is rapidly eliminated when PS II is exposed to light [33]. We investigated whether or not betaine could protect the ddPS II particles from inactivation by light. The ddPS II particles rapidly lost their oxygen-evolving activity during incubation at 0°C in the light. The presence of betaine did not have any significant effect on the inactivation of the ddPS II particles (data not shown).

The photoinactivation is caused by damage to components either on the acceptor or on the donor side of the photochemical reaction [34]. One of the major causes of photoinactivation is the proteolytic degradation of D1 proteins [35] and our data demonstrate that betaine has no protective effect against this type of inactivation of PS II.

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