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Glycinebetaine enhances and stabilizes the evolution of oxygen and the synthesis of ATP by cyanobacterial thylakoid membranes

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Glycinebetaine (betaine), an osmoregulant in halophilic plants, stabilized the evolution of oxygen and the synthesis of ATP by thylakoid membranes from the cyanobacterium *Synechocystis* PCC6803 when it was present during the preparation and incubation of the thylakoid membranes. Moreover, betaine enhanced the evolution of oxygen and the synthesis of ATP when present during assays. When betaine at 1.0 M was present during the preparation of thylakoid membranes and during the measurement of activity, the rate of evolution of oxygen was equivalent to that of intact cells.

Glycinebetaine; Photosynthetic oxygen evolution; Photosynthetic ATP synthesis; Stabilization of photosynthesis; *Synechocystis* PCC6803

1. INTRODUCTION

The cyanobacteria are prokaryotes that can perform oxygenic photosynthesis [1]. Their photosynthetic machinery contains 2 photosystems, and photosynthesis accompanied by the evolution of oxygen normally occurs in the same way as in the chloroplasts of higher plants and algae. The photosynthetic activities of thylakoid membranes isolated from cyanobacterial cells are more labile than those of chloroplasts from higher plants [2–5]. Therefore, considerable attention has been paid to the preparation of thylakoid membranes that are highly active in both electron transport and the synthesis of ATP [5–8].

Recently, we demonstrated that an osmoregulatory substance, glycinebetaine (betaine, hereinafter), protects the photosystem 2 complex of spinach from the salt-induced dissociation of extrinsic proteins and inactivation of the oxygen-evolving machinery [9,10]. In the present study we examined the effects of betaine during the preparation of thylakoid membranes from the cyanobacterium *Synechocystis* PCC6803 and found that betaine was effective both in the stimulation and in the protection of the oxygen-evolving machinery and the synthesis of ATP.

2. MATERIALS AND METHODS

2.1. *Preparation of thylakoid membranes*

Synechocystis PCC6803 was grown photoautotrophically at 34°C in medium BG 11 [11] supplemented with 1% CO_2 in air. Continuous illumination from incandescent lamps was provided at an intensity of 10 $W \cdot m^{-2}$. An exponential phase culture (1.5 l) was centrifuged at 5 000 × *g* for 10 min for collection of cells. The sedimented cells were washed twice by resuspension in 30 ml of a medium that contained 50 mM HEPES-NaOH (pH 7.5) and 30 mM $CaCl_2$, and centrifuged at 5 000 × *g* for 10 min. Cells were finally resuspended in 30 ml of 50 mM HEPES-NaOH (pH 7.5), 800 mM sorbitol and 1.0 M betaine (medium A, hereinafter), supplemented with 1 mM 6-amino-*n*-caproic acid. All subsequent steps in the preparation of membranes were performed at 4°C. The suspension was passed through a French pressure cell (40 K; SLM Instruments, Urbana IL, USA) at 160 MPa, with resultant disruption of about 75% of the cells. The homogenate was centrifuged at 10 000 × *g* for 10 min to remove unbroken cells, and the supernatant was centrifuged at 60 000 × *g* for 60 min. The sedimented thylakoid membranes were suspended in 30 ml of medium A and the suspension was centrifuged at 60 000 × *g* for 60 min. The collected thylakoid membranes were suspended in 0.4 ml of medium A and were kept at 4°C until use.

2.2. *Measurement of photosynthetic activities*

Photosynthetic evolution of oxygen was measured by monitoring the concentration of oxygen with a Clark-type oxygen electrode in a reaction medium that contained 50 mM tricine-NaOH (pH 7.5), 10 mM $CaCl_2$, 600 mM sucrose, 100 μM PBQ, 1.0 M betaine, and thylakoid membranes corresponding to 8 μg Chl·ml^{-1} [12]. The reaction mixture was incubated for 3 min before measurements were made. Red

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Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenol indophenol; DCMU, 3′,4′-dichlorophenyl-1,1-dimethylurea; DPC, diphenylcarbazide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); PMS, *N*-methylphenazonium methylsulfate; PBQ, phenyl-1,4-benzoquinone; Tricine, *N*-tris(hydroxymethyl)-methylglycine.

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actinic light at an intensity of 640 $W \cdot m^{-2}$ was provided from an incandescent lamp through a combination of a heat-absorbing optical filter (HA50; Hoya, Tokyo, Japan) and a red optical filter (R-60; Toshiba, Tokyo).

The reduction of DCIP in the light was determined by following the change in absorbance at 580 nm, with a reference beam of 500 nm, in a dual-wavelength spectrophotometer (UV-300; Shimadzu, Kyoto, Japan) [12]. The reaction medium was the same as that for assays of evolution of oxygen, except that PBQ was replaced by DCIP at 100 μM as the electron acceptor and DPC at 1.0 mM as the electron donor. The reaction mixture was incubated for 3 min before measurements were made. Red actinic light at an intensity of 1200 $W \cdot m^{-2}$ was obtained from an incandescent lamp through 2 optical filters as mentioned above.

Phosphorylation was assayed by a luciferin/luciferase method [13]. The reaction mixture contained 50 mM tricine-NaOH (pH 7.5), 600 mM sucrose, 10 mM NaCl, 4 mM K_2HPO_4, 5 mM $MgCl_2$ and 2 mM ADP. The reaction was performed under aerobic conditions for 1 min under illumination with red actinic light at an intensity of 1500 $W \cdot m^{-2}$ from an incandescent lamp through 2 optical filters as mentioned above.

The temperature was 24°C during the incubation of thylakoid membranes and the measurement of photosynthetic activities. Chl was determined by the method of Arnon et al. [14].

3. RESULTS

The absorption spectra of thylakoid membranes prepared from *Synechocystis* PCC6803 in the presence and absence of betaine had major peaks at 419, 439, and 680 nm and minor peaks at 495 and 625 nm. These characteristics of the absorption spectra suggest that phycobilins had been removed from the preparations of membranes [15].

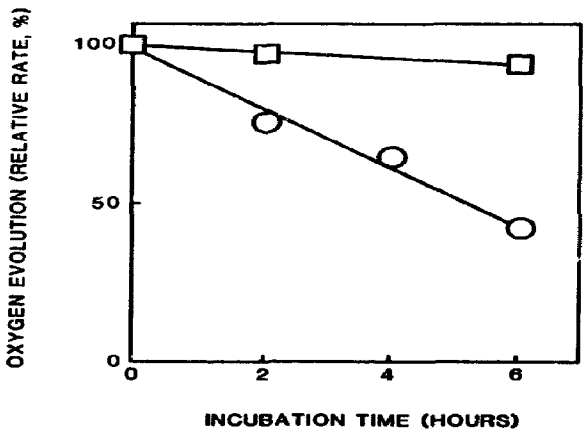

Fig. 1. Changes in oxygen-evolving activity during incubation in the presence or absence of betaine. Thylakoid membranes were prepared in the presence of 1.0 M betaine at all steps of preparation and storage. Membranes corresponding to 8 μg Chl·ml^{-1} were incubated at 24°C in 50 mM Tricine-NaOH (pH 7.5), 10 mM $CaCl_2$, and 600 mM sucrose, in the presence of 1.0 M betaine or in its absence. Oxygen-evolving activity was measured at 24°C after addition of PBQ at 100 μM to the incubation medium. The activity at 100% corresponded to 250 and 150 μmols O_2 (mg Chl)$^{-1} \cdot h^{-1}$ in the presence and absence of betaine, respectively. □, Betaine was present; ○, no betaine was present during incubation of membranes.

Table I

Effects of betaine on the oxygen-evolving activity of thylakoid membranes and intact cells

Type of preparation	Oxygen-evolving activity (μmols O_2 (mg Chl)$^{-1} \cdot h^{-1}$)			
	24°C		34°C	
	− Betaine	+ Betaine	− Betaine	+ Betaine
Thylakoid membranes prepared and stored with betaine	146 ± 11 (3)	237 ± 13 (3)	205 ± 19 (3)	321 ± 34 (3)
Thylakoid membranes prepared and stored without betaine	73 ± 40 (3)	75 ± 33 (3)	109 ± 10 (3)	112 ± 50 (3)
Intact cells	150 ± 22 (2)	154 ± 40 (2)	330 ± 10 (2)	322 ± 20 (2)

Thylakoid membranes were prepared either in the presence of 1.0 M betaine or in its absence at all steps of preparation and storage. The reaction medium contained 50 mM tricine-NaOH (pH 7.5), 10 mM $CaCl_2$, 600 mM sucrose, 100 μM PBQ, and thylakoid membranes corresponding to 8 μg Chl·ml^{-1}, in the presence of 1.0 M betaine or in its absence. The numerals in parentheses indicate the numbers of experiments. The evolution of oxygen was measured at 24°C and 34°C.

Table I shows the effects of betaine in the media used for preparation and assays on the evolution of oxygen by thylakoid membranes. Two types of protective effect of betaine were noted. The presence of betaine during the preparation of thylakoid membranes had a marked sustaining effect on oxygen-evolving capacity. The presence of betaine in the reaction medium had a substantial positive effect on the oxygen-evolving activity of thylakoid membranes prepared in the presence of betaine. No enhancement by betaine was observed in intact cells or in thylakoid membranes prepared in the absence of betaine.

Figure 1 shows the effects of betaine on the inactivation of the oxygen-evolving activity during incubation at 24°C of thylakoid membranes prepared in the presence of betaine. In the absence of betaine, the oxygen-evolving activity decreased by 50% after incubation for 6 h. In the presence of betaine at 1.0 M, in contrast, the oxygen-evolving activity remained constant for 6 h. These observations suggest that betaine markedly stabilizes the oxygen-evolving machinery.

The electron-transport reactions through photosystem 2 were assessed by monitoring the reduction of DCIP (Table II). Betaine significantly enhanced and stabilized the transport of electrons from H_2O to DCIP.

Table II

Effects of betaine on the capacity for and stability of the reduction of DCIP by thylakoid membranes

Duration of incubation at 24°C (h)	Electron-transport activity (μmols DCIP reduced (mg Chl)$^{-1}$·h^{-1})			
	$H_2O \rightarrow$ DCIP		DPC → DCIP	
	− Betaine	+ Betaine	− Betaine	+ Betaine
0	127 ± 6 (3)	219 ± 17 (3)	328 ± 29 (3)	320 ± 35 (3)
6	69 ± 4 (2)	220 ± 10 (3)	328 ± 20 (3)	340 ± 20 (3)

Thylakoid membranes were prepared in the presence of 1.0 M betaine at all steps of preparation and storage. The incubation medium contained 50 mM tricine-NaOH (pH 7.5), 10 mM $CaCl_2$, 600 mM sucrose and thylakoid membranes corresponding to 8 μg Chl·ml^{-1}, in the presence of 1.0 M betaine or in its absence. For measurements of electron- transport activity, 100 μM DCIP and 1.0 mM DPC were included in the medium. The numerals in parentheses indicate the numbers of experiments.

In the presence of DPC, which can serve as a donor of electrons to photosystem 2, such that the oxygen-evolving site is by-passed, the reduction of DCIP was not affected by betaine nor was it inactivated in either the presence or the absence of betaine. Thus, it can be concluded that the transport of electrons from DPC to DCIP is unaffected by incubation at 24°C and is not enhanced by betaine. These findings suggest that betaine stimulates and stabilizes the oxygen-evolving complex of photosystem 2, but it has practically no effect on other electron-transport reactions of photosystem 2, such as the photochemical separation of electric charge and the reduction of plastoquinone.

Table III presents the effects of betaine on the capacity for and the stability of the synthesis of ATP, which is coupled with the transport of electrons from H_2O to ferricyanide (non-cyclic phosphorylation) in thylakoid membranes prepared in the presence of betaine. Betaine was very effective in stimulating and stabilizing the synthesis of ATP. Table III also shows the effects of betaine on the activity and the stability of the synthesis of ATP that is coupled with the cyclic transport of electrons, mediated by PMS (cyclic phosphorylation). In this case, however, the stimulatory and stabilizing effects of betaine were not as marked as in the case of non-cyclic phosphorylation. This difference between the effects of betaine on the non-cyclic and cyclic phosphorylation reactions can be explained as follows. The non-cyclic phosphorylation is coupled with the transport of electrons from H_2O to ferricyanide, which must be activated by betaine as is that from H_2O to PBQ and DCIP. Thus, the non-cyclic phosphorylation can be doubly stimulated; directly by the effect of betaine on the synthesis of ATP and indirectly by the effect of betaine on the transport of electrons.

Table III

Effects of betaine on the capacity for and stability of non-cyclic and cyclic phosphorylation by thylakoid membranes

Duration of incubation (h)	Synthesis of ATP (μmols ATP formed (mg Chl)$^{-1}$·h^{-1})			
	Non-cyclic ($H_2O \rightarrow Fe(CN)_6$)		Cyclic (PMS)	
	− Betaine	+ Betaine	− Betaine	+ Betaine
0	243 ± 65 (3)	453 ± 50 (4)	357 ± 15 (4)	416 ± 63 (4)
6	93 ± 42 (4)	404 ± 73 (4)	249 ± 21 (3)	400 ± 37 (2)

Thylakoid membranes were prepared in the presence of 1.0 M betaine at all steps of preparation and storage. The incubation medium contained 50 mM tricine-NaOH (pH 7.5), 600 mM sucrose, 10 mM NaCl, 4 mM K_2HPO_4, 5 mM $MgCl_2$, 2 mM ADP, and thylakoid membranes corresponding to 8 μg Chl·ml^{-1}. Electron carriers were added as follows: 1.0 mM ferricyanide ($Fe(CN)_6$), for non-cyclic phosphorylation; and 25 μM PMS and 10 μM DCMU, for cyclic phosphorylation. The numerals in parentheses indicate the numbers of experiments.

4. DISCUSSION

In the present study, we discovered that betaine has dual effects on the evolution of oxygen and synthesis of ATP by thylakoid membranes prepared from *Synechocystis* PCC6803. (i) Betaine stabilizes these photosynthetic reactions when it is present during the preparation and incubation of membranes. (ii) Betaine stimulates the oxygen-evolving machinery and the synthesis of ATP. Thus, the evolution of oxygen and the synthesis of ATP occurred at high rates when betaine was present in the media used for preparation and assay of the membranes. The oxygen-evolving activity of the membranes was about the same as that of intact cells (Table I).

The mechanism responsible for the effects of betaine in stimulating and stabilizing the photosynthetic reactions of the cyanobacterial thylakoid membranes is not well understood. However, in previous studies [9,10], we demonstrated that betaine protects the photosystem 2 complex of spinach against the salt-induced dissociation of the extrinsic proteins from the complex, with the resultant stabilization of the capacity for the evolution of oxygen. Therefore, it is likely that betaine also protects the cyanobacterial oxygen-evolving complex from dissociation of the 33-kDa protein and/or 9-kDa protein [16–18]. We can predict that the thylakoid membranes prepared from *Synechocystis* PCC6803 in the present study were right-side-out closed vesicles since they were prepared without detergents. It seems likely that betaine penetrates the thylakoid membrane, entering the intra-thylakoid space and having access to the oxygen-evolving site of the photosystem 2 complex. It is also possible that betaine diffuses into the intra-thylakoid space as a result of the mechanical treatment during the disruption of the cells by the French-pressure treatment.

The most plausible explanation for the stimulation of the synthesis of ATP by betaine is that betaine stabilizes ATP synthase, a complex membrane protein composed of more than 10 polypeptides [19]. Another possibility is that the non-cyclic transport of electrons, mediated by PMS, is stimulated by betaine. Further studies are necessary to elucidate the molecular mechanism of the effects of betaine in stabilizing and stimulating the oxygen-evolving complex and the ATP synthase.

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