Glycinebetaine Counteracts the Inhibitory Effects of Salt Stress on the Degradation and Synthesis of D1 Protein during Photoinhibition in *Synechococcus* sp. PCC 7942

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Glycinebetaine (hereafter referred to as betaine) is a compatible solute that accumulates in certain plants and microorganisms in response to various types of stress. We demonstrated previously that when the cyanobacterium *Synechococcus* sp. PCC 7942 (hereafter *Synechococcus*) is transformed with the *codA* gene for choline oxidase, it can synthesize betaine from exogenously supplied choline, exhibiting enhanced tolerance to salt and cold stress. In this study, we examined the effects of salt stress and betaine synthesis on the photoinhibition of photosystem II (PSII). Salt stress due to 220 mM NaCl enhanced photoinhibition of PSII and betaine protected PSII against photoinhibition under these conditions. However, neither salt stress nor betaine synthesis affected photodamage to PSII. By contrast, salt stress inhibited repair of photodamaged PSII and betaine reversed this inhibitory effect of salt stress. Pulse-chase-labeling experiments revealed that salt stress inhibited degradation of D1 protein in photodamaged PSII and de novo synthesis of D1. By contrast, betaine protected the machinery required for degradation and synthesis of D1 under salt stress. Neither salt stress nor betaine affected levels of *psbA* transcripts. These observations suggest that betaine counteracts the inhibitory effects of salt stress, with resultant accelerated repair of photodamaged PSII.

Living organisms are frequently exposed to various kinds of environmental stress in their natural habitats and they have developed mechanisms that allow them to withstand such stress. One such mechanism involves the accumulation of compatible solutes, which are defined as water-soluble organic compounds of low molecular mass that are nontoxic at high concentrations (Chen and Murata, 2002).

Glycinebetaine (hereafter referred to as betaine) is a compatible solute; it is an amphoteric compound and extremely soluble in water. The molecular features of betaine allow it to interact with both the hydrophobic and the hydrophilic domains of macromolecules. Results of studies in vitro indicate that betaine stabilizes the structure and activity of enzymes and maintains the integrity of membranes against the damaging effects of excessive salt, cold, heat, and freezing (Gorham, 1995). Betaine is found in a wide variety of prokaryotes, eukaryotic microorganisms, higher plants, and animals (Wilken et al., 1970; Galinski and Trüper, 1982; Yancey et al., 1982; Mohammad et al., 1983; Hanson et al., 1985). In a comprehensive investigation of the actions of betaine in vivo, we transformed plants, namely, Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), and tomato (*Lycopersicon esculentum*), with the *codA* gene for choline oxidase, which catalyzes the synthesis of betaine from choline. The resultant transgenic plants synthesized and accumulated betaine and exhibited, in addition, enhanced tolerance to various environmental stresses, such as high salt, high and low temperatures, and freezing (Hayashi et al., 1997, 1998; Alia et al., 1998a, 1998b, 1999; Sakamoto and Murata, 2001a, 2001b ; Chen and Murata, 2002; Sulpice et al., 2003; Park et al., 2004).

We also transformed the cyanobacterium *Synechococcus* sp. PCC 7942 (hereafter *Synechococcus*) with the *codA* gene. The line of transformed *Synechococcus* cells, which we designated PAMCOD, synthesized betaine in vivo from exogenously supplied choline and accumulated betaine at levels of 60 to 80 mM (Deshnium et al., 1995). PAMCOD cells that are grown in the presence of 1 mM choline can continue to grow in the presence of 400 mM NaCl, whereas wild-type cells, which do not synthesize betaine from choline, cannot grow at all at this concentration of NaCl. PAMCOD cells exhibit similarly enhanced tolerance to low temperatures when supplied with exogenous choline. This enhanced tolerance to low temperature was ascribed to acceleration of the repair of photodamaged PSII and not to protection of PSII against photodamage (Deshnium et al., 1997).

In this study, we attempted to elucidate the mechanism whereby synthesis of betaine protects PAMCOD cells from salt stress. We found that betaine protected cyanobacterial cells against the synergistic actions of salt stress and strong light stress by accelerating repair of PSII. This acceleration was related to enhancement

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.106.076976.

of the degradation of D1 protein in photodamaged PSII and de novo synthesis of D1.

RESULTS

Betaine Protected *Synechococcus* against Photoinhibition of PSII under Salt Stress

In previous studies, we demonstrated that PAMCOD cells, namely, *Synechococcus* cells that had been transformed with the *codA* gene for choline oxidase, were able to synthesize betaine and accumulate this compatible solute in the cytosol at levels of 60 to 80 mM when choline was supplied exogenously as the substrate for betaine synthesis (Deshnium et al., 1995). Therefore, we examined the effects of strong light and 220 mM NaCl on PAMCOD cells grown in the absence of choline (control cells) and on PAMCOD cells grown in medium supplemented with 1.0 mM choline chloride, the substrate for betaine synthesis (choline-supplemented cells). Figure 1A shows that, when PAMCOD cells were exposed to strong light at 250 μmol photons m^{-2} s^{-1} under control conditions (20 mM NaCl), PSII activity decreased only slightly at the early stage of photoinhibition, but remained high subsequently. Synthesis of betaine, as a result of the availability of choline, did not affect photoinhibition of PSII under normal conditions.

Figure 1B shows that under salt stress due to the presence of 220 mM NaCl, photoinhibition of PSII was significantly enhanced. In particular, PSII activity decreased to 40% of the initial level during a 180-min incubation period. The apparent acceleration of photoinhibition under salt stress was consistent with our previous observations in *Synechocystis* sp. PCC 6803 (Allakhverdiev et al., 2002). In choline-supplemented cells, PSII activity fell to about 70% of the initial level during incubation in light at 250 μmol photons m^{-2} s^{-1} for 180 min. This result indicated that betaine, synthesized in vivo from exogenous choline, protected PSII against photoinhibition under salt stress.

Salt Stress Had No Effect on Photodamage to PSII But Inhibited Repair of PSII

Because the extent of photoinhibition depends on the balance between photodamage to PSII and repair of such damage, we postulated that it might be possible to explain the stimulation and repression of photoinhibition by salt stress and betaine synthesis, respectively, in terms of regulation of photodamage and/or repair. To identify the steps that were affected by the actions of salt stress and betaine, we examined photodamage and repair separately.

Photodamage to PSII can be examined separately from repair of PSII by use of lincomycin, an inhibitor of protein synthesis. Figure 1C shows that, in the presence of lincomycin, PSII activity declined rapidly during incubation in light at 250 μmol photons m^{-2} s^{-1} and that synthesis of betaine did not affect photodamage to PSII. Figure 1D shows that salt stress and synthesis of betaine did not affect the decrease in PSII activity that was due to photodamage. These results indicate that salt stress did not affect photodamage to PSII. Moreover, they were consistent with results obtained in *Synechocystis* (Allakhverdiev et al., 2002; Allakhverdiev and Murata, 2004) and, in addition, they showed that betaine did not protect PSII from photodamage.

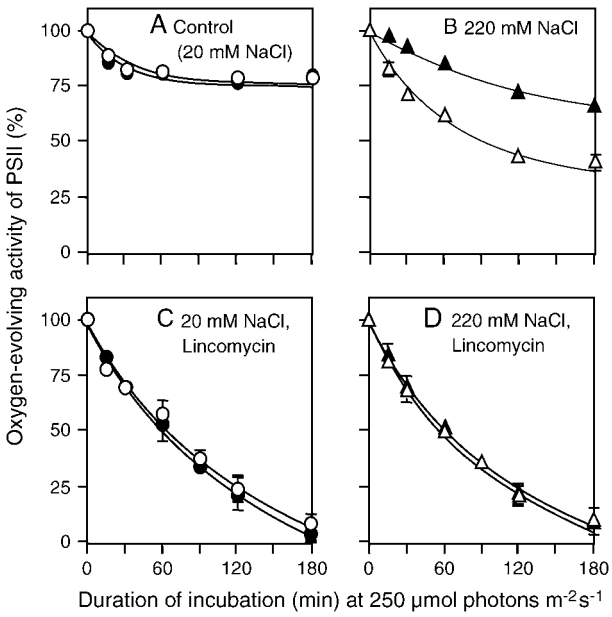

Figure 1. Betaine protected PSII against photoinhibition under salt stress in the PAMCOD line of *Synechococcus* cells. PAMCOD cells at 3 μg of chlorophyll mL^{-1} were exposed to strong white light at 250 μmol photons m^{-2} s^{-1} from an incandescent lamp in the presence of 20 mM NaCl (control conditions) or 220 mM NaCl (salt stress conditions) and in the presence or absence of 250 μg mL^{-1} lincomycin. To establish salt stress at 220 mM NaCl, a small portion of 5 M NaCl was added to cell cultures (i.e. 4% [v/v]) 3 min before exposure to strong light. PSII activity was measured by monitoring the oxygen-evolving activity in the presence of 1,4-benzoquinone as the artificial acceptor of electrons. The activity corresponding to 100% was 860 ± 34 μmol O_2 (mg chlorophyll)$^{-1}$ h^{-1}. White (○, △) and black (●, ▲) symbols represent control cells that had been unable to synthesize betaine and choline-supplemented cells that had accumulated betaine, respectively. A, Control (20 mM NaCl). B, 220 mM NaCl. C, 20 mM NaCl + 250 μg mL^{-1} lincomycin. D, 220 mM NaCl + 250 μg mL^{-1} lincomycin. Each point and error bar represents the average and SD, respectively, of results from three independent experiments.

The results in Figure 1 suggested that the protective effect of betaine on PSII activity might have been due to enhancement of the repair of PSII after photodamage. Therefore, we examined the effects of betaine synthesis on repair of PSII after photodamage. We exposed PAMCOD cells to strong light at 1,000 μmol photons m^{-2} s^{-1} for 60 min in the presence of 20 mM NaCl or for 45 min in the presence of 220 mM NaCl to reduce PSII activity to 30% of the initial level in each case, and then we transferred the cultures to weak light at 70 μmol photons m^{-2} s^{-1} for recovery (Fig. 2). In the absence of

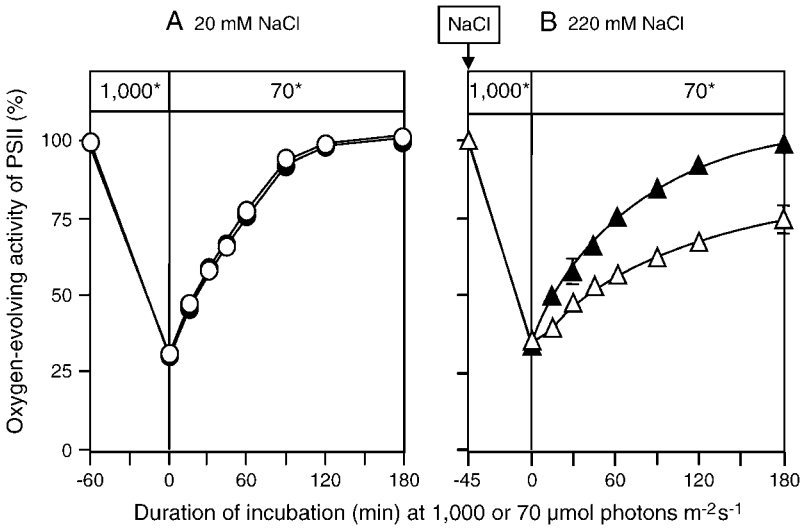

Figure 2. Betaine accelerated repair of photodamaged PSII under salt stress. PAMCOD cells were exposed to strong light at 1,000 μmol photons m^{-2} s^{-1} to decrease PSII activity to 30% of the original level and then transferred to weak light at 70 μmol photons m^{-2} s^{-1} to allow repair of photodamaged PSII. White (○, △) and black (●, ▲) symbols represent control cells that were unable to synthesize betaine and choline-supplemented cells that had accumulated betaine, respectively. Other experimental conditions were the same as described in the legend to Figure 1. A, Control (20 mM NaCl). B, Salt stress conditions (220 mM NaCl). The asterisk (*) indicates that the intensity of light is given in μmol photons m^{-2} s^{-1}. Each point and error bar represents the average and SD, respectively, of results from three independent experiments.

salt stress, PSII activity returned to the original level during incubation for 120 min and betaine did not affect this repair process (Fig. 2A). By contrast, salt stress significantly inhibited repair of PSII. During incubation in weak light for 180 min, PSII activity recovered to only approximately 70% of the initial level (Fig. 2B). However, the presence of betaine in choline-supplemented cells enhanced repair of PSII considerably, and complete recovery of PSII activity was observed after incubation for 180 min (Fig. 2B). Thus, the presence of betaine in the cytosol clearly reversed the inhibitory effects of salt stress on repair of photodamaged PSII.

Betaine Accelerated Degradation of D1 Protein under Salt Stress

Repair of photodamaged PSII involves several steps and degradation of D1 protein is the first of these steps. We examined the effects of salt stress and betaine on degradation of D1 in photodamaged PSII by pulse-chase analysis with radioactively labeled Met (Fig. 3). Figure 3A shows that, during incubation in light at 250 μmol photons m^{-2} s^{-1}, the level of pulse-labeled D1 decreased and the labeled protein almost disappeared during incubation for 180 min under control conditions. The level of labeled D1 in control cells decreased under salt stress more slowly than under control conditions, suggesting that salt stress due to 220 mM NaCl delayed degradation of D1 protein. By contrast, the decrease in the level of labeled D1 in choline-supplemented cells under salt stress was similar to that observed under control conditions.

The time course of the degradation of D1 protein (Fig. 3B) revealed that the rate of degradation of this protein was unaffected by the presence of intracellular betaine under control conditions. Under salt stress, there was an obvious decrease in the rate of degradation of D1 protein in control cells. However, this effect of 220 mM NaCl was barely evident in choline-supplemented cells. These observations suggest that salt stress inhibited degradation of D1 protein and that betaine counteracted the inhibitory effects of NaCl on degradation of this protein.

We also analyzed changes in the level of D1 protein by western blotting. It has been suggested that degradation and de novo synthesis of D1 protein might be coupled (Komenda and Barber, 1995). Thus, the inhibition by salt stress of the degradation of D1 might have been reflected by the inhibitory effect of NaCl on the de novo synthesis of D1. To evaluate this possibility, we estimated the rate of degradation of D1 protein by western-blot analysis under conditions where degradation was separated from de novo synthesis by inclusion of lincomycin. Figure 4 shows that, in the presence of lincomycin, the level of D1 decreased to approximately 20% of the original value during incubation in strong light for 180 min under control conditions (20 mM NaCl). The presence of betaine had no effect on the rate of degradation of D1 protein. Salt stress due to 220 mM NaCl significantly retarded degradation of D1 in control cells. Moreover, even in the presence of lincomycin, the rate of degradation of D1 under salt stress was increased in choline-supplemented cells. These observations confirmed the opposing effects of salt stress and betaine on degradation of D1 protein. They also suggested that the effects of salt stress and betaine on degradation of D1 are independent of the de novo synthesis of D1.

Betaine Accelerated de Novo Synthesis of D1 Protein under Salt Stress

We examined the effects of salt stress and betaine on de novo synthesis of D1 protein by pulse labeling with [^{35}S]Met. Figure 5A shows changes in the labeling patterns of thylakoid-membrane proteins during photoinhibition in the presence of 20 and 220 mM NaCl.

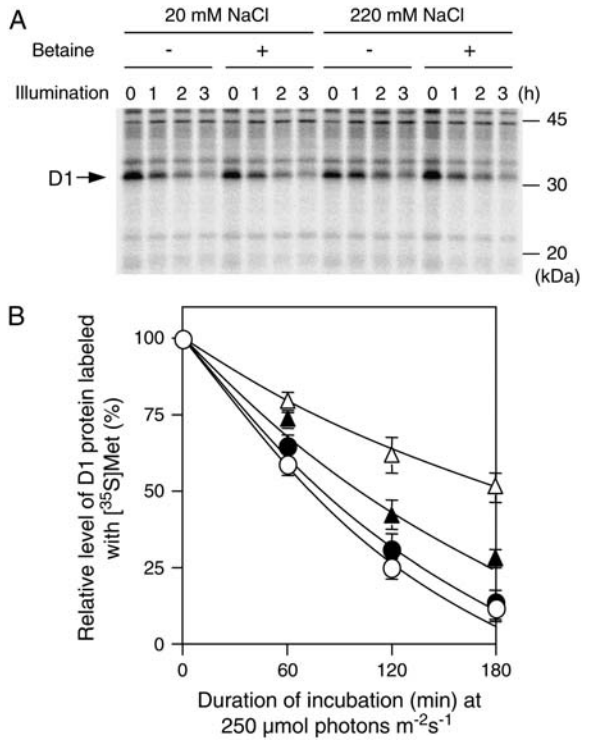

Figure 3. Betaine accelerated degradation of D1 protein in photodamaged PSII under salt stress (pulse-chase experiment). PAMCOD cells were pulse labeled with [^{35}S]Met and radioactivity was pulse chased during exposure to light at 250 μmol photons m^{-2} s^{-1}, as described in "Materials and Methods." Thylakoid membranes were isolated from cells that had been harvested at designated times and the proteins in the thylakoid membranes were solubilized and separated by SDS-PAGE as described in "Materials and Methods." A, Autoradiogram. B, Time course of changes in levels of D1 protein that had been labeled with [^{35}S]Met during incubation in light at 250 μmol photons m^{-2} s^{-1}. ○, Control cells (20 mM NaCl); ●, choline-supplemented cells (20 mM NaCl); △, control cells (220 mM NaCl); ▲, choline-supplemented cells (220 mM NaCl). Each point and error bar represents the average and SD, respectively, of results from three independent experiments.

Under control conditions (20 mM NaCl), de novo synthesis of thylakoid-membrane proteins was unaffected by the presence of exogenous choline. By contrast, salt stress (220 mM NaCl) clearly decreased the levels of radioactively labeled proteins. However, in choline-supplemented cells, radioactively labeled proteins in thylakoid membranes remained at levels similar to those observed under control conditions. The time course of changes in the level of radioactively labeled D1 (Fig. 5B) showed that betaine did not affect de novo synthesis of D1 in light under nonstress conditions (20 mM NaCl). By contrast, under salt stress (220 mM NaCl), synthesis of D1 was significantly inhibited. However, the presence of choline improved the efficiency of synthesis of D1. These results suggest that salt stress inhibits de novo synthesis of D1 and that betaine reverses the inhibitory effects of salt stress on synthesis of D1 protein.

We examined the effects of salt stress and betaine on the level of *psbA* transcripts by northern blotting. The genome of *Synechococcus* includes three *psbA* genes, designated *psbAI*, *psbAII*, and *psbAIII*. Whereas only the *psbAI* gene is expressed under normal conditions in light at 70 μmol photons m^{-2} s^{-1}, expression of the *psbAII* and *psbAIII* genes is induced and that of *psbAI* mRNAs is repressed upon exposure of *Synechococcus* cells to strong light at 250 μmol photons m^{-2} s^{-1} (Golden et al., 1986; Kulkarni et al., 1992). The results of northern blotting indicated that such changes in the expression of the *psbAI*, *psbAII*, and *psbAIII* genes occurred under all sets of conditions examined, suggesting that neither salt stress nor betaine synthesis

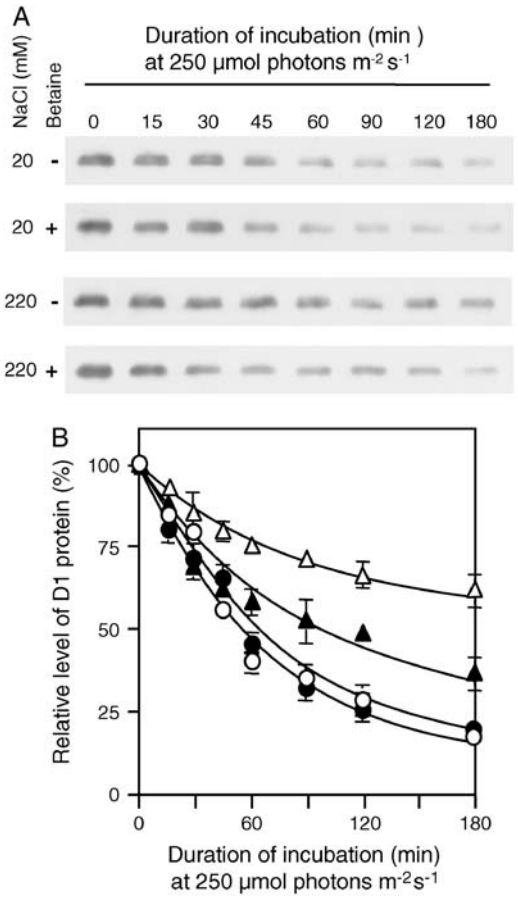

Figure 4. Betaine accelerated degradation of D1 protein in photodamaged PSII under salt stress. PAMCOD cells were exposed to light at 250 μmol photons m^{-2} s^{-1} in the presence of 250 μg mL^{-1} lincomycin. Thylakoid membranes were isolated from cells that had been harvested at designated times. Proteins in thylakoid membranes were solubilized and separated by SDS-PAGE as described in "Materials and Methods." After blotting onto a nitrocellulose membrane, the proteins were allowed to react with D1-specific antibodies. A, Western blot of D1 protein. B, Time course of changes in levels of D1 protein during incubation in light at 250 μmol photons m^{-2} s^{-1}. ○, Control cells (20 mM NaCl); ●, choline-supplemented cells (20 mM NaCl); △, control cells (220 mM NaCl); ▲, choline-supplemented cells (220 mM NaCl). Each point and error bar represents the average and SD, respectively, of results from three independent experiments.

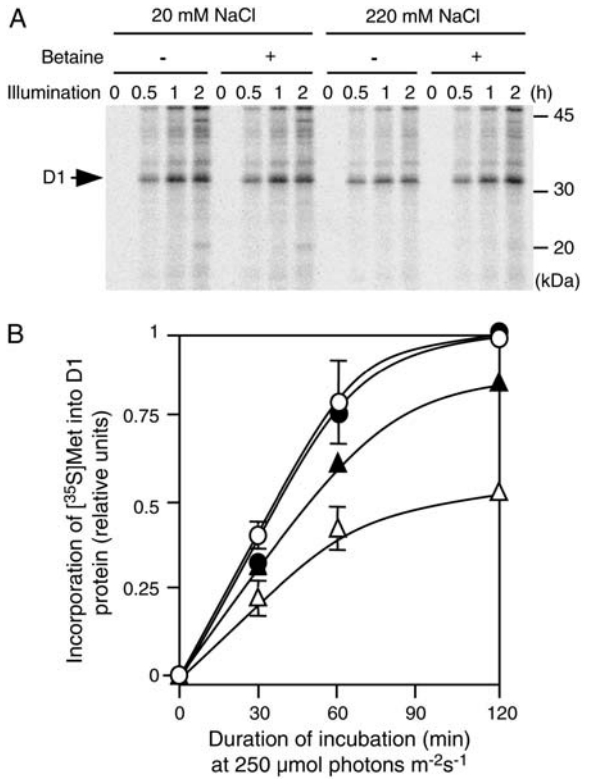

Figure 5. Betaine accelerated de novo synthesis of D1 protein during photoinhibition under salt stress. PAMCOD cells were labeled with [^{35}S]Met during incubation in light at 250 μmol photons m^{-2} s^{-1} as described in "Materials and Methods." Thylakoid membranes were isolated from cells that had been harvested at designated times and proteins in thylakoid membranes were solubilized and separated by SDS-PAGE as described in "Materials and Methods." A, Autoradiogram. B, Time course of changes in levels of D1 protein that had been labeled with [^{35}S]Met during incubation in light at 250 μmol photons m^{-2} s^{-1}. ○, Control cells (20 mM NaCl); ●, choline-supplemented cells (20 mM NaCl); △, control cells (220 mM NaCl); ▲, choline-supplemented cells (220 mM NaCl). Each point and error bar represents the average and SD, respectively, of results from three independent experiments.

affected the transcription of *psbA* genes (data not shown). Therefore, we can conclude that the inhibition and enhancement of synthesis of the D1 protein, as shown in Figure 5, were not caused by any effects of salt stress and betaine synthesis on transcription of the *psbA* genes.

DISCUSSION

Hypothetical Scheme for the Effects of Salt Stress and Betaine on Photoinhibition

In this study, we demonstrated that salt stress due to 220 mM NaCl repressed repair of photodamaged PSII by inhibiting degradation and synthesis of D1 protein. We also demonstrated that the presence of exogenously supplied choline reversed the inhibitory effect of 220 mM NaCl on repair of PSII by accelerating degradation and de novo synthesis of D1 protein. It is reasonable to assume that this effect of choline is related to the synthesis and accumulation of betaine in PAMCOD cells, namely, transformed *Synechococcus* cells that express choline oxidase, synthesize betaine from choline, and accumulate betaine at levels of 60 to 80 mM (Deshnium et al., 1995). Moreover, the protective effect of betaine appeared when repair of PSII was repressed by salt stress.

Figure 6 shows a hypothetical scheme for the actions of NaCl and betaine. Photodamage to D1 protein, which can be isolated from the repair process by lincomycin, was unaffected by the presence of NaCl and betaine. Only degradation of D1 protein and translation of *psbA* mRNAs are influenced by NaCl and betaine.

Possible Mechanisms for the Effects of Salt Stress on Degradation and de Novo Synthesis of D1 Protein

Degradation of D1 protein is one of the earliest in the repair of photodamaged PSII. Genetic and biochemical approaches have demonstrated that two proteases are

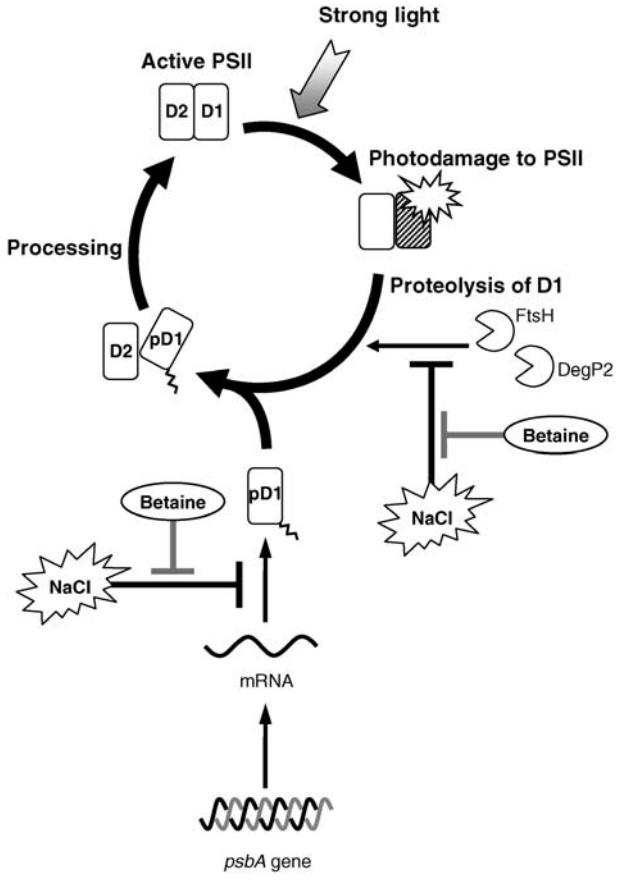

Figure 6. Hypothetical scheme for the effects of salt stress and betaine on the photodamage-repair cycle of PSII during photoinhibition. In this scheme, salt stress inhibits both degradation of D1 protein and de novo synthesis of the pre-D1 (pD1). Betaine reverses these inhibitory effects of salt stress, with resultant enhancement of the repair of PSII under salt stress conditions.

involved in this degradation, namely, FtsH (Lindahl et al., 2000; Silva et al., 2003) and DegP2 (Haußühl et al., 2001; Silva et al., 2002). The two proteases probably act in concert to degrade D1. This study demonstrated that salt stress due to 220 mM NaCl inhibits degradation of D1 in *Synechococcus* (Figs. 3 and 4). The molecular mechanism responsible for such inhibition is unclear. However, it seems possible that salt stress might inhibit interaction between these proteases and D1 or that salt stress might inactivate these proteases. It has been reported that FtsH participates in the formation of large protein complexes of approximately 500 kD in *Synechocystis* sp. PCC 6803 (Silva et al., 2003). A homolog of FtsH in Arabidopsis, VAR2, which is involved in degradation of D1 protein (Bailey et al., 2002), forms a protein complex of 400 to 450 kD with VAR1, which is another homolog of FtsH (Sakamoto et al., 2003). Salt stress might dissociate the constituents of such large complexes. DegP2 is bound to the stromal side of thylakoid membranes and is released from isolated thylakoid membranes by treatment with NaCl (Haußühl et al., 2001). Salt stress might dissociate DegP2 from thylakoid membranes in *Synechococcus* with resultant inhibition of degradation of D1 protein.

De novo synthesis of D1 protein is an important event in the repair of PSII (Aro et al., 1993). Synthesis of D1 requires transcription of *psbA* genes, translation of *psbA* transcripts to yield the precursor to D1 protein (pre-D1), incorporation of pre-D1 into the PSII complex, and processing of pre-D1 to generate mature D1 protein. In a previous study of *Synechocystis* sp. PCC 6803, we demonstrated that salt stress due to 500 mM NaCl inhibits translation of *psbA* transcripts, but does not affect transcription of *psbA* genes (Allakhverdiev et al., 2002). In this study, we found that salt stress due to 220 mM NaCl inhibited translation of *psbA* transcripts, but did not affect transcription of *psbA* genes in *Synechococcus*. The difference with respect to the concentration of NaCl that is effective in the two cyanobacteria might be related to the fact that *Synechococcus* is more sensitive to salt stress than *Synechocystis* sp. PCC 6803.

Translation of *psbA* transcripts is a strongly regulated process and many regulatory factors are probably involved in both initiation and elongation. In yeast (*Saccharomyces cervisiae*), initiation of translation involves a variety of regulatory factors, such as eukaryotic initiation factor (eIF) 3 and eIF4E (Pyronnet and Sonenberg, 2001), and initiation is a target of inhibition by salt stress (Uesono and Toh-E, 2002). The genome of *Synechococcus* includes genes for the translation initiation factors of prokaryotic type, namely, IF1 and IF2 (GenBank accession no. NC_006576; available at cyano.genome.jp). Therefore, a similar inhibitory action of salt stress might occur in *Synechococcus* cells. Salt stress might inhibit the interaction of nucleotides with the regulatory protein complex and/or the formation of functional protein complexes.

Peptide elongation is also controlled at several steps (Kim et al., 1991; Tyystjärvi et al., 2001) and, in *Synechococcus*, one such step is the targeting of the *psbA* mRNA-ribosome complex to the thylakoid membrane (Tyystjärvi et al., 2001). Under strong light and low-temperature stress, *psbAII* and *psbAIII* mRNAs are translated, whereas *psbAI* mRNA is not, when these transcripts are associated with membrane-bound ribosomes (Tyystjärvi et al., 2004). Thus, elongation during translation probably involves a number of regulatory factors and the elongation step could also be a site of inhibition by salt stress. It remains to be determined whether it is the initiation step or the elongation step in the translation of *psbA* transcripts that is inhibited by salt stress in *Synechococcus*. Identification of factors that are most sensitive to salt stress requires further investigation of the mechanism that regulates synthesis of D1 protein.

Betaine Counteracts the Effects of Salt Stress on Degradation and de Novo Synthesis of D1 Protein

Betaine counteracted the effects of NaCl on degradation of D1 protein (Fig. 4). As shown in Figures 3 and 5, we observed the effects of betaine and NaCl not only on the turnover of D1 protein, but also on that of some other proteins that bind to thylakoid membranes; these proteins might have contributed, in part, to the repair of the PSII complex. Therefore, it seems likely that both NaCl and betaine might affect the proteolytic and translational machinery. Opposing effects of betaine and NaCl have also been observed in the synthesis of soluble proteins in nonphotosynthetic bacteria, such as *Staphylococcus aureus* (Vijaranakul et al., 1997) and *Enterococcus faecalis* (Pichereau et al., 1999).

NaCl impedes the intermolecular association of protein subunits and betaine might compete with NaCl and enhance such an association. The integrity and activity of the translational machinery might be reduced by salt stress and enhanced by betaine. There is ample evidence that betaine stabilizes the conformation of proteins and, in particular, the quaternary structure of complex proteins. For example, betaine protects the oxygen-evolving complex of PSII from the NaCl- and heat-induced dissociation of extrinsic proteins (Papageorgiou and Murata, 1995).

Two hypotheses have been proposed for the mechanism whereby betaine stabilizes macromolecular structures. One hypothesis involves the formation of a hydration shell (layers of bound water) around the surface of proteins (Arakawa and Timasheff, 1983) and the other involves the direct stabilization of proteins by betaine (Schobert, 1977). In *Synechococcus*, betaine might be able to stabilize proteases and the translational machinery by one or both mechanisms even under salt stress.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Synechococcus sp. PCC 7942 cells were transformed with the *codA* gene for choline oxidase (X84895) as described previously (Deshnium et al., 1995) and

the resultant mutant cells were designated PAMCOD cells. They were grown at 34°C in BG11 medium that contained 20 mM NaCl in the presence of 1.0 mM choline chloride (choline-supplemented cells) or in its absence (control cells) under illumination from incandescent lamps at 70 μmol photons m^{-2} s^{-1} with aeration by air that contained 1% CO_2. Cells were grown to a concentration that corresponded to approximately 5 μg chlorophyll mL^{-1}. An aliquot was withdrawn and diluted to a chlorophyll concentration of 3 μg mL^{-1} with BG11 medium that contained 1.0 mM choline chloride or no choline. After incubation for 60 min under the same conditions as above, cells were used for experiments.

Measurement of PSII Activity

We measured PSII activity at 34°C in BG11 medium in terms of the photosynthetic evolution of oxygen by monitoring the concentration of oxygen with a Clark-type oxygen electrode (Hansatech Instruments) in the presence of 1 mM 1,4-benzoquinone as the electron acceptor (Ono and Murata, 1981). Actinic light at 1,500 μmol photons m^{-2} s^{-1} was provided by an incandescent lamp equipped with a heat-absorbing filter (HA-50; Hoya Glass) and a red optical filter (R-60; Toshiba).

Preparation of Thylakoid Membranes

Four-milliliter aliquots of suspensions of cells at 3 μg chlorophyll mL^{-1} were withdrawn from cultures at designated times during incubation in strong light and centrifuged in Eppendorf tubes at 15,000*g* for 10 min. Each cell pellet was suspended in 180 μL of 50 mM Tris-HCl (pH 8.0) and 330 mg of glass beads (106 μm; Sigma-Aldrich) were added. The cells were disrupted by vortexing for 5 min with 30-s intervals between each minute of vortexing. The homogenate was transferred to a new Eppendorf tube with 1 mL of 50 mM Tris-HCl (pH 8.0) and then subjected to centrifugation at 15,000*g* for 10 min. The resultant pellet of thylakoid membranes was resuspended in 60 μL of 50 mM Tris-HCl (pH 8.0) that contained 25% (w/v) glycerol.

Western-Blot Analysis

Proteins in thylakoid membranes were solubilized by incubation at 65°C for 5 min in the presence of 2% SDS and 0.1 M dithiothreitol (2.5 μL of a mixture of 10% SDS and 0.5 M dithiothreitol were added to 10 μL of a suspension of thylakoid membranes that corresponded to 1 μg of chlorophyll). The solubilized proteins were separated by SDS-PAGE on a 15% polyacrylamide gel that contained 6 M urea. Separated polypeptides were blotted electrophoretically onto a nitrocellulose membrane and probed with polyclonal antibodies raised in rabbits against the full-length D1 protein from spinach (*Spinacia oleracea*; Vermass et al., 1988). Signals were visualized with an enhanced chemiluminescence reagent (ECL; Amersham Biosciences). Antibodies raised against D1 protein were kindly provided by Dr. M. Ikeuchi (University of Tokyo).

Pulse-Labeling and Pulse-Chase Experiments

Synechococcus cells that had been grown to a concentration that corresponded to approximately 5 μg chlorophyll mL^{-1} were collected by centrifugation at 7,500*g* for 10 min at 30°C and then resuspended in fresh BG11 medium. Cells resuspended in BG11 medium at 3 μg chlorophyll mL^{-1} were incubated at 34°C for 1 h in light at 70 μmol photons m^{-2} s^{-1} with aeration by air that contained 1% CO_2.

In pulse-labeling experiments, cells were subsequently exposed to light at 250 μmol photon m^{-2} s^{-1} with stirring by a magnetic stirrer in the presence of 5 nM [^{35}S]Met (5 μCi mL^{-1}; Amersham Biosciences). In pulse-chase experiments, after incubation with aeration as above, cells were incubated at 34°C for 10 min in light at 70 μmol photons m^{-2} s^{-1} with stirring by a magnetic stirrer. After a 10-min incubation, cells were supplemented with 5 nM [^{35}S]Met and incubated for another hour under the same conditions as above. Immediately prior to exposure to light at 250 μmol photons m^{-2} s^{-1}, nonradioactive Met was added to a final concentration of 5 mM.

Pulse labeling and pulse chase were terminated by addition of lincomycin at 250 μg mL^{-1} and immediate cooling of samples on ice. Thylakoid membranes were isolated as described above and the membranes equivalent to 10 ng of chlorophyll were fractionated by SDS-PAGE on a 15% polyacrylamide gel that contained 6 M urea. Labeled proteins were detected with a Bio-imaging analyzer (BAS2000; Fuji Photo Film). Levels of labeled D1 protein were estimated densitometrically.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number X84895.

ACKNOWLEDGMENT

We thank Dr. M. Ikeuchi of the University of Tokyo for providing antibodies against the full-length D1 protein.

Received January 19, 2006; revised April 15, 2006; accepted April 17, 2006; published April 21, 2006.

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