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Glycinebetaine stabilizes photosystem 1 and photosystem 2 electron transport in spinach thylakoid membranes against heat inactivation

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Abstract

Glycinebetaine, a compatible osmolyte of halotolerant plants and bacteria, partially protected photosystem (PS) 1 and PS2 electron transport reactions against thermal inactivation but with different efficiencies. In its presence, the temperature for half-maximal inactivation ($t_{1/2}$) was generally shifted downward by 3-12 °C. Glycinebetaine stabilized photoinduced oxygen evolving reactions of PS2 by protecting the tetranuclear Mn cluster and the extrinsic proteins of this complex. A weaker, although noticeable, stabilizing effect was observed in photoinduced PS2 electron transport reactions that did not originate in the oxygen-evolving complex (OEC). This weaker protection by glycinebetaine was probably exerted on the PS2 reaction centre. Glycinebetaine protected also photoinduced electron transport across PS1 against thermal inactivation. The protective effect was exerted on plastocyanin, the mobile protein in the lumen that carries electrons from the integral cytochrome b_6f complex to the PS1 complex.

Additional key words: oxygen exchange; *Spinacia oleracea*; temperature stress.

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Abbreviations: Chl - chlorophyll; Cyt - cytochrome; DAD - diaminodurene; DCIP - 2,6-dichlorophenolindophenol; DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DQH_2 - duroquinol; DPC - diphenylcarbazide; ddPS2 - particles of PS2 without 33, 24, and 17 kDa extrinsic proteins; HEPES - N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid); Mes - 4-morpholinethanesulfonic acid; MV - methylviologen; OEC - oxygen-evolving complex; PC - plastocyanin; PBQ - phenyl-*p*-benzoquinone; PMS - phenazine methosulfate; PS - photosystem; Q_A and Q_B - primary and secondary quinone acceptors of PS2, respectively; RC - reaction centre; Tricine - N-tris(hydroxymethyl)-methylglycine.
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Introduction

High temperatures destroy photosynthetic functions of higher plants due to a number of thermally-induced processes that include: (*1*) Dissociation of the peripheral chlorophyll-protein antenna complexes from PS2 core complexes and destacking of grana (Armond *et al.* 1980, Gounaris 1984). (*2*) Inactivation of the OEC (Berry and Björkman 1980, Nash *et al.* 1985, Thompson *et al.* 1989). (*3*) Alterations in the $Q_A \rightarrow Q_B$ electron transfer at the acceptor site of PS2 (Bukhov *et al.* 1990). The PS2 complex is the most labile segment of the photosynthetic apparatus (Al-Khatib and Paulsen 1989, Srivastava *et al.* 1997). In contrast, the PS1 complex resists elevated temperatures more effectively (Thomas *et al.* 1986, Boucher and Carpentier 1993).

Glycinebetaine (hereinafter betaine) is an amphiphilic zwitterion accumulating in photosynthetic cells of higher and microbial plants, that protects the tetranuclear Mn cluster and extrinsic proteins of the OEC from chaotropic denaturation by high concentrations of NaCl and other electrolytes (Papageorgiou *et al.* 1991, Murata *et al.* 1992, Mohanty *et al.* 1993, Rajasekaran *et al.* 1997) as well as against thermal inactivation (Mamedov *et al.* 1991, 1993). But there has been no report on the stabilization by betaine of photoinduced electron transport reactions that do not involve photosynthetic oxygen evolution. Yet more recent data favour a protective effect by betaine of oxygen evolution-independent PS2 electron transport (Allakhverdiev *et al.* 1996).

In the present study we examined systematically the protective effect of betaine against the thermal inactivation of photoinduced PS2 and PS1 electron transport reactions, using unstacked spinach thylakoids and PS2 and PS1 membrane particles.

Materials and methods

Unstacked thylakoids isolated from spinach leaves (*Spinacea oleracea* L.) according to Steinback *et al.* (1979) were stored in liquid nitrogen. Thylakoid membranes were prepared and stored in solutions containing 0.5 M betaine. The thylakoid membranes were incubated for 10 min at designated temperatures in darkness, in a medium that contained 25 mM Hepes NaOH (pH 7.5), 400 mM sucrose, and 10 mM NaCl, either in the absence or presence of betaine. The suspension was then cooled down to 20 °C and after the addition of electron transport cofactors, photosynthetic electron transport was measured.

Membrane particles enriched in PS2 activity (PS2 particles) were isolated from spinach leaves as described by Berthold *et al.* (1981). Their oxygen evolution activity was 167 $mmol(O_2)\ kg^{-1}(Chl)\ s^{-1}$. To obtain modified PS2 particles that lacked all three extrinsic proteins, samples of PS2 membrane particles were incubated in a medium consisting of 1 M $CaCl_2$, 300 mM sucrose, 25 mM Mes-NaOH, pH 7.0 (Ono and Inoue 1984). Membrane particles enriched in PS1 were isolated according to Boardman (1971). Plastocyanin was isolated from spinach leaves as described by Morand (1993), with minor modifications. Plastocyanin was collected on a *Toyopearl HW-60* column in the presence of ammonium sulfate and eluted in the absence of ammonium sulfate.

Oxygen evolution or uptake by suspensions of thylakoid membranes and PS2- or PS1-enriched membrane particles was measured by monitoring the concentration of oxygen with a Clark-type oxygen electrode. "White light" (160 W m^{-2}) was provided to samples from a 150 W tungsten-halogen source. $H_2O \rightarrow$DCIP and H_2O/DPC$\rightarrow$ DCIP photoinduced electron transport was measured by DCIP photoreduction with a single-beam absorption spectrophotometer according to Kuwabara and Murata (1983). DCIP photoreduction was quantified in terms of absorbance decrease at 600 nm. The chlorophyll (Chl) content of samples was determined according to Arnon (1949). Thylakoid membranes were added to a final concentration that corresponded to 5-10 g(Chl) m^{-3}. Duroquinone was reduced according to Izawa and Pan (1978) immediately before use.

Results

Results will be discussed by reference to the simplified photosynthetic electron transport sequence shown below. Only relevant intermediates are indicated. Immobile electron carriers, embedded in integral membrane complexes, are enclosed within brackets. Artificial electron donors appear above the main electron transport sequence (shown in bold symbols), artificial electron acceptors below it. The reaction centres for PS2 (P680) and for PS1 (P700) are indicated in italics.

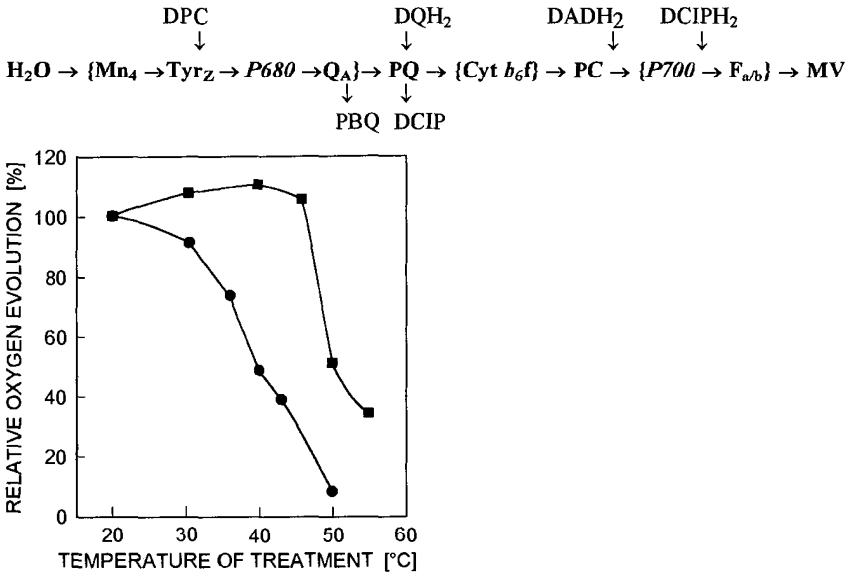

Fig. 1. Effect of 0.5 M betaine (■) on the thermal inactivation of PBQ-dependent photosynthetic oxygen evolution by spinach thylakoid membranes. Thylakoid suspensions with 10 kg(Chl) m^{-3} in 25 mM Hepes.NaOH, 10 mM NaCl, 400 mM sucrose, pH 7.5, were incubated at designated temperatures for 10 min and then they were cooled to 20 °C. After addition of 0.4 mM PBQ the electron-transport activity was measured by monitoring of oxygen evolving. ● - no betaine added to both the preparation and incubation mixtures.

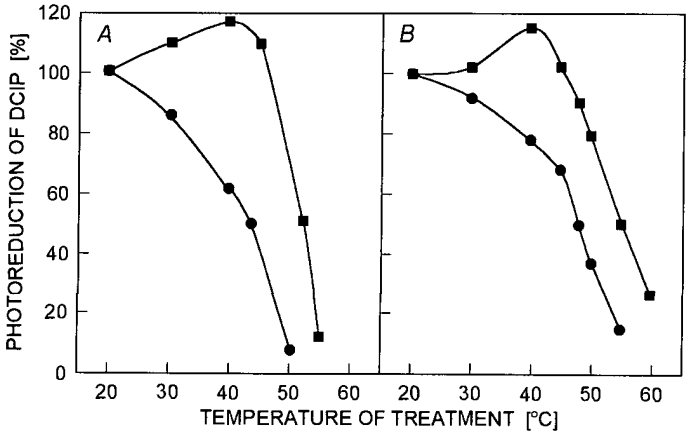

Fig. 2. Effect of 0.5 M betaine (■) on the thermal inactivation of the $H_2O \rightarrow$DCIP (*A*) or DPC→DCIP (*B*) photoreduction by spinach thylakoid membranes. Conditions as in Fig. 1. After the addition of 0.05 mM DCIP and 0.5 mM DPC the electron-transport activity was measured by monitoring the photoreduction of DCIP. ● - no betaine added to both the preparation and incubation mixtures.

Temperature affects the electron transport from H_2O to PBQ in suspensions of unstacked thylakoid membranes (Fig. 1). PBQ accepts electrons from PS2 exclusively (Renger *et al.* 1988, Iwaki and Itoh 1989). The segment comprises immobile carriers integrated in the D_1D_2 heterodimer (*i.e.*, the OEC and the PS2 photoreaction complex). The rate of photoinduced electron transport was quantified in terms of oxygen evolution. In the absence of betaine, inactivation of oxygen evolution became appreciable above 30 °C, and the activity was lost gradually up to *ca.* 50 °C. By contrast, in the presence of betaine, oxygen evolution increased gradually up to *ca.* 40 °C, but then it dropped abruptly. The protective effect of the betaine was apparent in the $t_{1/2}$, the half-maximal inactivation temperature of oxygen evolution. In the betaine-free suspensions it was at 39 °C, while in the betaine-containing suspensions at about 50 °C. Analogous results were reported by Mamedov *et al.* (1993) for cell-free thylakoid membranes of the cyanobacterium *Synechocystis* sp. PCC 6803.

Fig. 2 shows the temperature inactivation curves for DCIP photoreduction with H_2O (*A*) or DPC (*B*) as electron donors. DPC couples to Tyr_Z, on the oxidizing side of PS2 (*i.e.*, it bypasses the Mn cluster of the OEC). DCIP photoreduction in the presence of DPC reflects competing electron donations by H_2O and by DPC. Electron transport $H_2O \rightarrow$DCIP by unstacked thylakoids (Fig. 2*A*) *in vitro* responded to temperature similarly as the PBQ-dependent electron transport except for a shift of $t_{1/2}$ (Fig. 1*A*). In the absence of betaine, the $t_{1/2}$ of the $H_2O \rightarrow$DPC electron transport was at 44 °C, while in the presence of betaine $t_{1/2}$ it was at 53 °C. According to Fig. 2*B* and to the $t_{1/2}$ values, electron transport H_2O/DPC→DCIP was more stable at increasing temperature than both the $H_2O \rightarrow$PBQ and the $H_2O \rightarrow$DCIP electron transports. This probably reflects the fact that in DPC it couples to Tyr_Z, *i.e.*, it bypasses the heat-labile OEC, but DPC may also reduce DCIP chemically.

The obtained values for the rate of DPC→DCIP (Fig. 2*B*) indicate that the PS2 complexes in the thylakoid membranes retain almost all the reaction centres active for oxygen evolution. Betaine was capable of stabilizing the reaction probably by affecting the RC of PS2 at those high temperatures when OEC was inactivated (Fig. 2*B*).

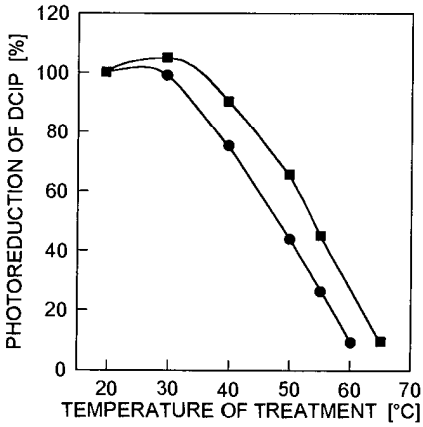

Fig. 3. Effect of 0.5 M betaine (■) on the thermal inactivation of DCIP photoreduction by ddPS2 particles with DPC as electron donor. Conditions as in Fig. 1. After the addition of 0.05 mM DCIP and 0.5 mM DPC the electron-transport activity was measured by monitoring the photoreduction of DCIP. ● - no betaine added to both the preparation and incubation mixtures.

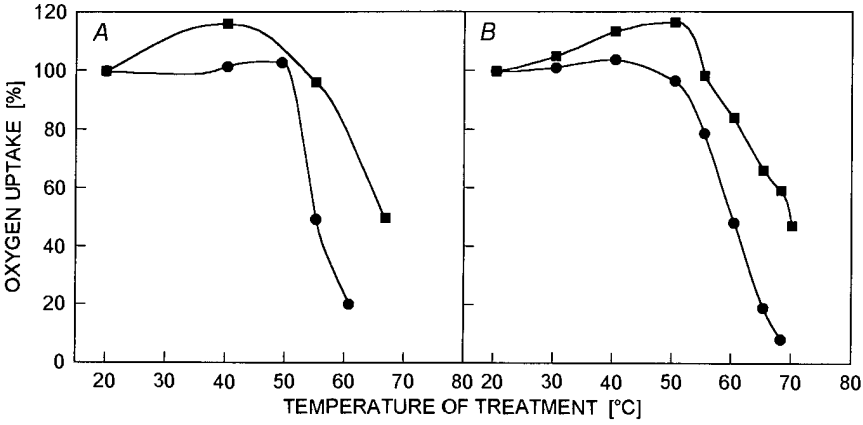

Fig. 4. Effect of 1.0 M betaine (■) on the thermal inactivation of the MV-mediated photoinduced uptake of oxygen by spinach thylakoid membranes with DQH_2 (*A*) and $DADH_2$ (*B*) as electron donors. Conditions as in Fig. 1. After the addition 0.01 mM DCMU, 0.2 mM MV, 0.5 mM DQH_2 (*A*) or 0.5 mM DAD, 1 mM Asc-Na (*B*) electron-transport activity was measured by monitoring the uptake of oxygen. ● - no betaine added to both the preparation and incubation mixtures.

To confirm that betaine is effective in protecting and stimulating the electron transport reactions of PS2 complexes at heat stress, we analyzed the effect of betaine on the DCIP photoreduction in ddPS2 particles that had lost all peripheral proteins

maintaining OEC. There was a stabilizing effect of betaine on electron transport reactions also with ddPS2 particles (Fig. 3). Moreover, a 50 % inactivation of electron transport activity with ddPS2 particles took place at higher temperatures than in membrane preparations. These results may be explained by a greater thermostability of RC PS2 than of the OEC in general. Betaine shifted the $t_{1/2}$ of the DCIP photoreduction from 49 to 55 °C. Thus under the heat stress betaine protects not only the OEC but also stabilizes the reactions close to RC PS2.

Fig. 4 shows the temperature dependence of the heat inactivation of $DQH_2 \rightarrow MV$ and $DADH_2 \rightarrow MV$ in the presence of DCMU in unstacked thylakoid membranes, with or without betaine. MV accepts electrons from $F_{a/b}$, the iron-sulfur centre on the acceptor side of the PS1 complex (Parrett *et al.* 1989). Duroquinol functions as a donor to plastoquinone in a DBMIB-sensitive PS1 electron transfer reaction. These activities (Fig. 4*A*) were, in contrast to the whole-chain and PS2 electron transport, more tolerant to heat treatment. The $t_{1/2}$ values were 55 and 63 °C in the absence and presence of betaine, respectively.

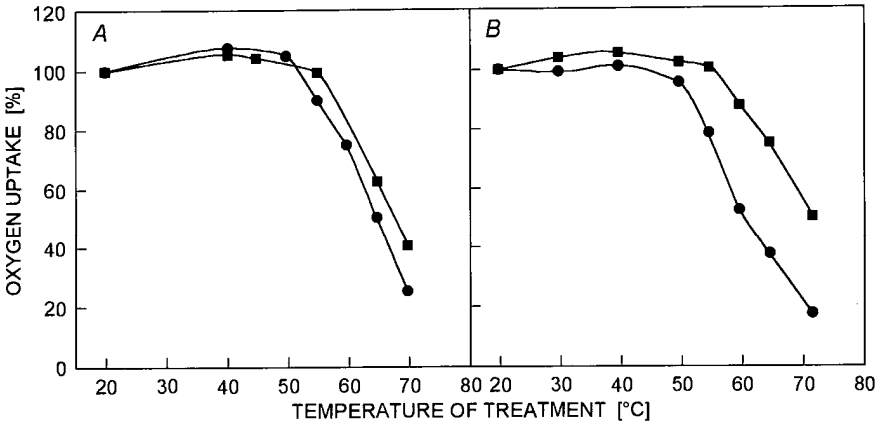

Fig. 5. Effect of 1.0 M betaine (■) on the thermal inactivation of the MV-mediated photoinduced uptake of oxygen by isolated PS1 particles with $DCIPH_2$ (*A*) and $DADH_2$ (*B*) as electron donors. PS1 particles at 5 kg(Chl) m^{-3}, suspended in 25 mM Tricine-NaOH, 20 mM KCl, pH 7.0, were incubated at designated temperatures for 10 min and then they were cooled to 20 °C. After the addition of 0.2 mM MV, 1 mM Asc-Na, 0.05 mM $DCIPH_2$ or 0.5 mM $DADH_2$ the electron-transport activity was measured by monitoring the uptake of oxygen. ● - no betaine added to both the preparation and incubation mixtures.

The temperature dependence of inactivation of the PS1 activity measured as oxygen uptake with ascorbate-reduced DAD as electron donor is demonstrated in Fig. 4*B*. PS1 activity may be destroyed by heat treatment, but the temperature stability of PS1 was much higher than those of PS2 (Fig. 2*B*) and PS1 with DQH_2 as electron donor. The oxidation site in $DADH_2 \rightarrow MV$ reaction is a soluble electron carrier protein, plastocyanin (Izawa 1980). The stabilizing effect of betaine in this reaction was stronger than in the DQH system. The $t_{1/2}$ in the absence of betaine was about 61 °C, while in the presence of betaine the rate of oxygen uptake by PS1

increased slightly along with temperature until a maximum was reached at about 50 °C and $t_{1/2}$ was at 72 °C. The difference between both treatments for the $DQH_2 \rightarrow MV$ reaction, where the Cyt b_6f complex is predominantly included in electron transport chain, was only 8 °C.

Our results demonstrate that betaine stabilizes not only PS2 reactions but also electron transport reactions within PS1. We compared the effect of betaine on the PS1 electron transfer reactions of isolated PS1 particles (Fig. 5*A*) and in the presence of exogenously added PC (Fig. 5*B*). A very slight protection of the PS1 electron transfer by betaine in the PS1 particles was found under high temperatures. However, the stabilizing effects of betaine were not as marked as in the case of thylakoid membranes. The slight stabilizing effect of betaine on the donation from reduced DCIP directly to P700 (Izawa *et al.* 1973) points to actions of a protector on the donor side of PS1.

However, the inactivation of temperature dependence and betaine protection of PS1 particles reconstituted with PC (Fig. 5) was similar to the results obtained with unstacked thylakoid membranes. The PS1 particles reconstituted with PC preserved 60 % activity even at 70 °C if betaine was present, while in the absence of betaine only 20 % of activity remained at 70 °C.

Discussion

Thylakoid membranes and multiprotein assemblies of the PS1 and particular PS2 reactions are thermally unstable, easily susceptible to structural randomization and functional inactivation upon heating (Thompson *et al.* 1989, Bukhov *et al.* 1990). The main target for inactivation of photosynthetic electron transport activity by heat has been generally considered to be the PS2 complex. The PS2 is unique with respect to other photosynthetic multiprotein complexes in that it not only performs light-induced charge separation but also can oxidize water to molecular oxygen. Thermal inactivation of oxygen evolution has been correlated with the release of functional Mn from PS2 together with the loss of three extrinsic polypeptides of 33, 24, and 17 kDa associated with oxygen evolution (Nash *et al.* 1985). Betaine stabilizes O_2 evolution by PS2 particles from higher plants (Papageorgiou *et al.* 1991) and cyanobacteria (Stamatakis and Papageorgiou 1993) against salt-induced inactivation. Initial suggestion on the mechanism of action of betaine was that it prevents the release of Mn ions from the OEC (Mohanty *et al.* 1993). Almost the same was concluded using the results obtained with *Synechocystis* sp. PCC6803 thylakoid membranes (Mamedov *et al.* 1993). Betaine is partly hydrophobic and zwitterionic. Local electrostatic interactions between zwitterions and proteins are perhaps important for maintaining the Mn cluster in an active form. The presence of betaine is beneficial only to the reactions of cyanobacterial photosynthesis under heat stress that involve the OEC (Mamedov *et al.* 1993, Papageorgiou and Murata 1995). Betaine shifts $t_{1/2}$ of the oxygen-evolving machinery of *Synechocystis* sp. PCC6803 thylakoids from 36 to 42 °C (Mamedov *et al.* 1993).

Allakhverdiev *et al.* (1996) showed that not only oxygen evolving activity but also the PS2 RC activity is affected by betaine. Results of Figs. 2*A,B* and 3 show a betaine effect on RC PS2 activity at high temperature when OCE became inactivated. The results obtained with ddPS2 particles (Fig. 3) confirm the stabilizing effect of betaine on the reactions near PS2 RC including probably the acceptor side of this photosystem. Mamedov *et al.* (1991) report that betaine protects PMS-mediated cyclic photophosphorylation driven by PS1 from the harmful effects of prolonged incubation at 34 °C. In this connection, our results on the stabilizing effect of betaine on the rate of oxygen uptake by PS1 complexes (Figs. 4*A,B* and 5) are reasonable. Moreover, stimulation of oxygen uptake does not occur with donors that act at the level of PC but is observed with donors acting at the oxidation site in the Cyt b_6f complex (see Boucher and Carpentier 1993). Hence in the $DADH_2 \rightarrow MV$ reaction, PC is mainly involved in the oxidation site (see Figs. 4 and 5). Betaine is more effective with regard to the heat-induced destabilization of soluble proteins (Laurie and Steward 1990). According to our results, this osmolyte along with specific effects on the oxygen-evolving complex (see Papageorgiou and Murata 1995) demonstrates "co-solute" properties and probably acts on the soluble electron transfer protein at the donor side of PS1. This stabilization effect of betaine is understandable in terms of theory of Arakawa and Thimasheff (1985): it suggests that co-solutes such as betaine might minimize the plastocyanin-water interaction (see Papageorgiou and Murata 1995).

Our present study demonstrates that betaine is capable to protect and stimulate the electron transport in unstacked spinach thylakoid membranes within PS2 and PS1 complexes.

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