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Effect of Trehalose on Oxygen Evolution and Electron Transfer in Photosystem 2 Complexes

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Abstract—The pigment–protein complex of photosystem 2 (PS 2) catalyzes the light-driven oxidation of water molecule and the reduction of plastoquinone. In this work, we studied the effect of the disaccharide trehalose, which is unique in its physicochemical properties, on isolated PS 2 complex. It was found that trehalose significantly stimulated the steady-state rate of oxygen evolution. The study of single flash-induced fluorescence decay kinetics demonstrated that trehalose did not affect the rate of Q_A^- oxidation, although it led to an increase in the relative fractions of PS 2 reaction centers capable of Q_A^- oxidation. Trehalose also prevented PS 2 complexes from being inactivated on prolonged storage. We propose that in the presence of trehalose, which affects the extent of hydration, the protein can preferentially exist in a more optimal conformation for effective functioning.

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The major source of energy in the biosphere is sunlight. However, only oxygenic photosynthetic organisms are able to convert the energy of photons emitted by the Sun into the energy of chemical bonds of carbohydrates by splitting water into molecular oxygen and reducing equivalents (electrons and protons). The pigment–protein complex of photosystem 2 (PS 2) embedded into the thylakoid membranes of cyanobacteria and chloroplasts functions as a light-driven water-plastoquinone oxidoreductase [1, 2]. All the organic and inorganic redox-active cofactors involved in charge transfer reactions are believed to reside on the D1 and D2 subunits of the reaction center (RC). A Mn_4CaO_5 cluster together with its coordinating amino acids and four water molecules form a catalytic site where oxidation of water molecules occurs [3-7]. It should be noted that the water-oxidizing complex (WOC), which is located on the donor side of the enzyme, is the most fragile site within PS 2 and is easily susceptible to oxidative damage.

The use of osmolytes in the stabilization of biomolecules is an old trick of Nature ([8-13] and references therein). In the presence of these small molecules, photosynthetic organisms counteract various stress conditions that they encounter. The osmolytes range from sugars to polyols, amino acids and their derivatives, and so forth. Among these, in recent years trehalose, which is naturally produced by several species of eubacteria, archaea, some fungi, certain invertebrates, and lower plants, has received considerable attention [14, 15].

The specific physical and chemical characteristics of trehalose such as relative inertness of the glycosidic linkage, the existence of both crystalline and amorphous states, thermostability (melting point, 203°C), high glass transition temperature, high stability over a wide pH range (3.5-10.0), and high hydrophilicity (water solubili-

Abbreviations: DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; P680, primary electron donor; PS 2, photosystem 2; Q_A and Q_B, primary and secondary quinone acceptors; RC, reaction center; WOC, water-oxidizing complex; Y_Z, redox-active tyrosine 161 of D1 protein.

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ty, 68 g/100 ml) set it apart from other osmolytes such as sucrose or glycerol.

The protective effect of trehalose on the photosynthetic apparatus under abiotic stresses (against dehydration, high salinity, and heat stress) has been demonstrated in transgenic plants ([16, 17] and references therein). Earlier, it was shown that trehalose prevents the release of soluble plastocyanin from thylakoid membranes during freeze–thaw treatment [18]. It was also shown that trehalose protected the chloroplast membranes and PS 2 particles during long and short freezing times [19]. It should be noted that the coupling between electron transfer and protein dynamics has been investigated in detail in photosynthetic bacterial reaction centers incorporated into glassy trehalose matrices [10, 20]. The data suggest that the conformational change stabilizing charge separation between the primary donor ($P870^+$) and the reduced primary quinone acceptor (Q_A^-) is localized around the Q_A binding pocket.

In the present work, we studied the effect of trehalose on electron transfer reactions within PS 2 particles from spinach. The data show that this disaccharide significantly stimulates the activity of the water-oxidizing complex of PS 2 and prevents time-dependent inactivation of the protein.

MATERIALS AND METHODS

Oxygen-evolving PS 2 core complexes and PS 2 membrane fragments from spinach were prepared as described in [21] and [22], respectively.

The rate of oxygen evolution under continuous illumination (1000 μmol photon·m^{-2}·s^{-1}) was measured at 25°C using a Clark-type oxygen electrode in medium containing 25 mM MES-NaOH (pH 6.5), 15 mM NaCl, 10 mM $CaCl_2$, 1 mM $K_3[Fe(CN)_6]$, and 0.1 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) unless otherwise stated.

Fluorescence transients of PS 2 core particles were measured using an FL3000 fluorometer (Photon Systems Instruments, Czech Republic). The sample was illuminated by continuous light at 625 nm with the intensity at the surface of the cuvette of 2000 μmol photon·m^{-2}·s^{-1}.

To monitor the fluorescence decay after a single saturating flash, actinic and the measuring light pulses were produced by red light-emitting diodes. The duration of the flash was 10 μs. Samples were assayed in medium containing 25 mM MES-NaOH (pH 6.5), 15 mM NaCl, and 10 mM $CaCl_2$ in the absence and in the presence of 1 M trehalose. All samples were dark incubated for 5 min prior to measurement.

The degree of the protein degradation was determined by the pellet content.

Kinetic signals were analyzed using the Origin program package (OriginLab Corporation, USA).

RESULTS AND DISCUSSION

The steady-state oxygen evolving activity of PS 2 complexes was monitored in the presence of trehalose (Fig. 1). The rate of oxygen evolution increased from 825 (in control samples) up to 2060 μmol O_2·(mg Chl·h)$^{-1}$ in the presence of 1 M trehalose in PS 2 core complexes (Fig. 1a). In the case of membrane fragments, the rate of oxygen evolution increased from 740 to 1340 μmol O_2·(mg Chl·h)$^{-1}$, correspondingly (Fig. 1b).

After 2 min of illumination of PS 2 core complexes in the absence of trehalose, the rate of oxygen evolution decreased ~35% in comparison with the initial rate (Fig. 1a, trace *1*). In so doing, the decrease in the rate of O_2 evolution was ~10% and ~7.5% in the presence of 0.5 M (trace *2*) and 1 M trehalose (trace *3*), respectively. Similar analysis of the rate of O_2 evolution kinetics in case of membrane fragments (Fig. 1b, trace *1*) showed that in the absence of trehalose the rate of oxygen evolution in PS 2 cores after 2 min illumination of the samples decreased by ~15% in comparison with the initial rate. After 2 min illumination, but in the presence of 0.5 M trehalose (Fig. 1b, trace *2*), the rate of O_2 evolution decreased by ~5%, while there was no decrease in the presence of 1 M trehalose (Fig. 1b, trace *3*).

These data indicate that trehalose stabilizes the water-oxidizing complex. It should be noted that further increase in trehalose concentration was not possible due to its limited solubility at room temperature [23].

For further investigations of the effect of trehalose, only PS 2 core complexes were used. These purified complexes transfer electrons from water to the terminal quinone acceptor as well as evolve oxygen. It should be noted that in the presence of 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) the rate of O_2 evolution was largely diminished (~75% inhibition) (data not shown).

The fluorescence transients of intact PS 2 core particles, just like in the case of PS 2 membrane fragments [24-28], are characterized by a biphasic (OJ and JP) kinetic pattern. In general, the initial OJ (photochemical) phase reflects the extent of reduction of the primary quinone acceptor Q_A, whereas the JP phase kinetics and amplitude are a measure of the rate and extent of the reduction of the terminal plastoquinone Q_B at the PS 2 acceptor side.

Figure 2 shows the fluorescence induction kinetics of dark-adapted PS 2 core particles in the absence (trace *1*) and in the presence of 1 M trehalose (trace *2*). Addition of trehalose increased the JP-phase amplitude, which is related to an increase in PS 2 RCs capable of reducing the terminal quinone acceptor.

We also investigated the effect of trehalose on the stability of electron transfer within PS 2 complex. Figure 2 demonstrates that 30-day storage of PS 2 core complexes at 4°C resulted in a significant (~2.3-fold) decrease in the

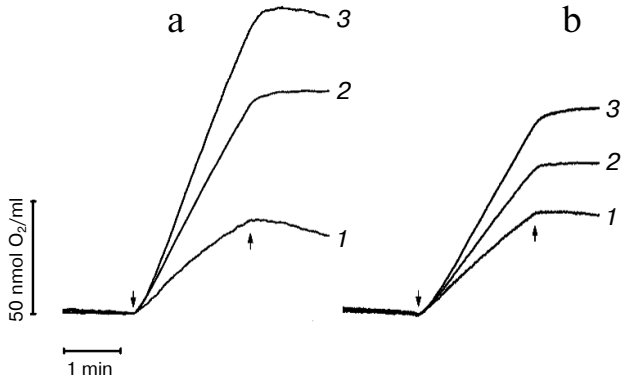

Fig. 1. Effect of trehalose on oxygen evolution in PS 2 core complexes (a) and PS 2 membrane fragments (b) in medium containing 25 mM Mes (pH 6.5), 15 mM NaCl, and 10 mM $CaCl_2$ in the absence (*1*) or presence of 0.5 M (*2*) or 1 M (*3*) trehalose. $K_3[Fe(CN)_6]$ (1 mM) and DCBQ (0.1 mM) were used as electron acceptors. The concentration of chlorophyll in the samples was 2 μg/ml. The rate of oxygen evolution (μmol O_2·(mg Chl·h)$^{-1}$)) in PS 2 core particles: *1*) 825; *2*) 1500; *3*) 2060; in membrane fragments: *1*) 740; *2*) 1030; *3*) 1340. ↓ and ↑ designate light (λ = 650 nm, 1000 μmol photon·m^{-2}·s^{-1}) on and off, respectively.

OJ and more dramatic (~6-fold) decrease in the JP phases (the JP/OJ ratio diminishes by a factor of ~2.5) (cf. traces *1* and *3*). The decrease in the amplitude in the OJ phase is due to lessening of the number of centers capable of Q_A reduction because of protein inactivation. The more pronounced decrease in the JP phase indicates, at first glance, that the acceptor side of the complex is more sensitive to the storage than the donor side. However, a similar decrease in the OJ phase and the JP/OJ ratio means that the latter parameter directly correlates with the number of centers with functional WOC.

The storage of PS 2 complexes in the presence of trehalose at 4°C for 30 days led to a significant increase in both the OJ and JP phases (4- and 3.7-fold, respectively) compared to the samples in the absence of trehalose stored under the same conditions (cf. traces *3* and *4*). It should be noted that under these conditions in the presence of trehalose degradation of proteins was not observed (not shown). However, the JP/OJ ratio remained similar in the absence and in the presence of trehalose (it decreased by less than ~10%). This result indicates that trehalose equally prevents damage to the donor and the acceptor sides of electron transfer in PS 2. Note also that almost 2-fold increase in the OJ phase amplitude after 30-day storage in the presence of trehalose (trace *4*) compared to the control (signal after 5 min dark adaptation in the absence of trehalose) (trace *1*) means that the fractions of PS 2 RCs capable of Q_A reduction increases after storage. In fact, the time-dependent degradation of the PS 2 complex results in the extraction of the Mn ions from its binding sites and the corresponding shift of the redox-potential of Q_A/Q_A^- from low- to high-potential form, which leads to a decrease in the equilibrium constant of electron transfer from Q_A^- to Q_B [29]. However, storage of samples for 30 days in the presence of trehalose retained oxygen-evolving activity only in ~20% of centers in comparison with control samples. On the other hand, in addition to a stabilizing role of trehalose at the level of the WOC, its mode of action could also include direct protection and/or stabilization of the RC itself. The maintenance of similar JP/OJ ratio suggests that the extent of Q_A^- oxidation by Q_B depends mainly on the number of centers with functional RC. Thus, we suggest that storage of PS 2 in the presence of trehalose partially prevents the inactivation of the WOC, but it mostly stabilizes the RC structure. Earlier, it was shown that addition of glycinebetaine to RC complex (D1/D2/Cyt b_{559}) protects the capacity for reduction of Cyt b_{559} from $Pheo^-$ and subsequent re-oxidation of Cyt b_{559} by $P680^+$, i.e. cyclic electron flow around PS 2 against photo- and heat-induced inactivation [30]. We propose a similar mechanism for the effect of trehalose on stabilization of PS 2 core complexes during storage of samples.

Redox reactions of quinone acceptors in PS 2 were almost exclusively characterized by measurements of chlorophyll fluorescence decay after single turnover light flashes [31-37]. In dark-adapted samples, the reduction of the primary quinone acceptor Q_A after a single flash gives rise to an increased fluorescence from PS 2, which decays as Q_A^- is reoxidized [34-37]. In the present work, we measured the reoxidation of Q_A^- to study the kinetics of electron transfer from Q_A^- to the secondary electron quinone acceptor Q_B in the absence (curve *1*) and in the

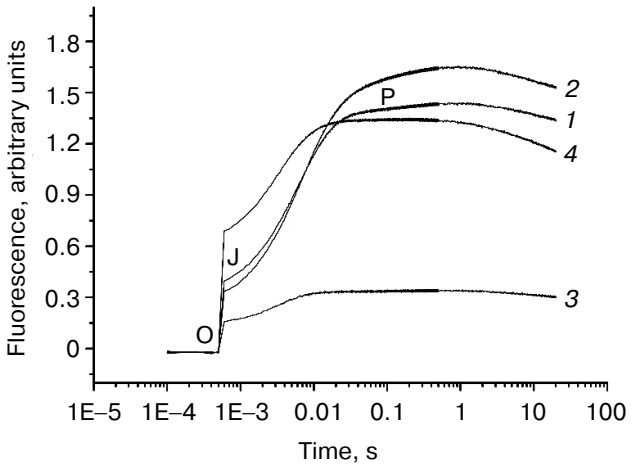

Fig. 2. Fluorescence transients of PS 2 core particles in the absence (*1*) and presence (*2*) of 1 M trehalose. Storage of dark-adapted samples during 30 days at 4°C in the absence (*3*) and in the presence (*4*) of 1 M trehalose. The sample, in a 2 ml cuvette, was illuminated by continuous light at 625 nm with intensity at the surface of the cuvette 2000 μmol photon·m^{-2}·s^{-1}. The concentration of chlorophyll in the samples was 10 μg/ml. The assay medium as in Fig. 1.

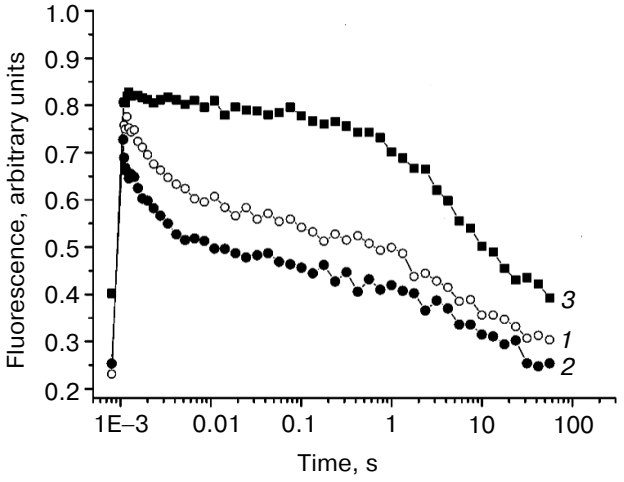

Fig. 3. Effect of trehalose on the kinetics of Q_A^- reoxidation after a single saturating flash to dark-adapted PS 2 core complexes. The decay of Chl fluorescence in the absence (*1*) and the presence (*2*) of 1 M trehalose; *3*) conditions as in (*1*), but in the presence of 5 μM DCMU. Assay medium as in Fig. 1. For other conditions, see "Materials and Methods".

presence (curve *2*) of 1 M trehalose (Fig. 3). In PS 2 complex, Q_A^- can be reoxidized by two pathways: due to forward electron transfer to Q_B and due to recombination with the oxidized components on the donor side.

Note that recombination reactions between Q_A^- and the S-states in the WOC or the redox-active tyrosine Y_Z (in Mn-depleted samples) occur much slower (>50 ms) than the forward electron transfer [5]. Forward electron transfer from Q_A^- to Q_B was found to be heterogeneous and has to be described with at least two time constants of 0.2-0.8 and 2-3 ms [4, 31-34]. Additionally, this first electron transfer step is influenced by several factors, e.g. pH, dehydration, and temperature [38]. In PS 2 core complexes, the flash-induced fluorescence decay was multiphasic (table). The kinetics were approximated by three exponential components with the following lifetimes (τ): τ_1 ~ 1.6 ms (relative contribution ~52%), τ_2 ~ 67 ms (~13%), and τ_3 ~ 5.5 s (~35%). The fast phase reflects Q_A^- oxidation by Q_B, while the two slower kinetic phases can be ascribed to back electron transfer from Q_A^-. The 67-ms phase represents the fraction of PS 2 RCs that lack a functional Mn_4CaO_5 cluster, in which Q_A^- recombines with oxidized redox-active tyrosine Y_Z [36, 37]. The slow phase (τ_3) is likely due to recombination between Q_A^- and the positively charged species in the WOC in PS 2 centers that are not able to perform the forward electron transfer.

In the presence of trehalose, the flash-induced fluorescence decay was also approximated by three kinetic phases: τ_1 ~ 1.4 ms (relative contribution ~64%), τ_2 ~ 110 ms (~13%), and τ_3 ~ 10 s (~23%) (table). The results obtained in the absence (Fig. 3, curve *1*) and in the presence of trehalose (Fig. 3, curve *2*) do not reveal significant difference in the lifetimes of these kinetic phases. The main effect of the trehalose is the ~12% increase in the relative contribution of the fast phase (τ_1) corresponding to forward electron transfer from Q_A^- at the expense of the slowest phase (τ_3) attributable to recombination reaction between Q_A^- and the S_2 state of the Mn_4CaO_5 cluster. The data suggest that trehalose facilitates forward electron transfer from Q_A^-.

By measuring the flash-induced fluorescence decay kinetics in the presence of DCMU, it is possible to examine the electron transfer on the donor side of PS 2 [36, 37, 39]. DCMU binds to the Q_B site and inhibits forward electron transfer from Q_A^-. Under these conditions, the recombination kinetics between Q_A^- and the positively charged donor side components (after a single flash, the main component is the S_2 state in the WOC) reflects the

Kinetic parameters for Q_A^- reoxidation after a single flash in PS 2 core complexes

Experimental conditions	τ_1, ms	τ_2, ms	τ_3, s	τ_4, s
No additions	1.6 ± 0.07 (52% ± 3)	67 ± 5 (13% ± 2)	5.5 (35% ± 2)	
+ 1 M trehalose	1.4 ± 0.1 (64% ± 3)	110 ± 3 (13% ± 4)	10 (23% ± 3)	
– trehalose			2	12
+ 5 μM DCMU			(33% ± 3)	(67% ± 2)
+ 1 M trehalose			1.5	12
+ 5 μM DCMU			(40% ± 5)	(60% ± 3)

Note: % in brackets represent relative contribution of the kinetic component to the total fluorescence decay.

integrity of the electron transfer chain on the oxidizing side of PS 2 [36, 37]. Under these conditions, the fast phase corresponding to the forward Q_A^- reoxidation reaction disappeared, and the kinetics was approximated by slow components in the time range of seconds (Fig. 3, curve *3*). The reason for the lack of an intermediate phase (τ_2) in the presence of inhibitor (table) is not clear. The phase with $\tau_3 \sim 2$ s (~33%) is likely due to recombination of Q_A^- with partially reduced Mn centers, while the very slow phase (~12 s, ~67%) probably corresponds to PS 2 RCs that were in the S_0 state before the flash [40, 41]. It should be noted that, upon addition of trehalose, the time constant and the contribution of the flash-induced fluorescence decay phases in the presence of DCMU (table), which reflect charge recombination between Q_A^- and oxidizing-side components of the PS 2, remained almost the same.

The mechanism of the effect of trehalose on PS 2 complex is not clear. It is known that in governing specific dynamics and functions of proteins, water molecules belonging to the protein hydration shell, to the bulk solvent, or placed inside the protein are critical [10, 20]. Data at extremely low hydration in bacterial RC suggest that a limited number of tightly bound water molecules stabilize the primary light induced charge-separated state on the time scale of 10^{-2} s [42].

Thus, in this work we have shown that addition of trehalose significantly stimulates the steady-state rate of O_2 evolution in both PS 2 membrane fragments and core complexes. By the analysis of the initial rates of oxygen evolution, it was shown that trehalose stabilizes the oxidation of water, and these data are in good agreement with the results obtained by FTIR spectroscopy of isolated PS 2 complex [43].

As in the case of sucrose and glycerol [35], trehalose has no effect on the Q_A^- reoxidation kinetics, although it stimulates this reaction in a greater fraction of PS 2 RCs capable of oxidizing Q_A^-. As noted before, trehalose also prevents the time-dependent degradation and inactivation of PS 2 samples, probably as a result by stabilization of the reaction center. We propose that in the presence of trehalose, which affects the extent of hydration, the protein can preferentially exist in a more optimal conformation for effective functioning.

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