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Zwitterion Modulation of O_2-Evolving Activity of Cyanobacterial Photosystem II[†]

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ABSTRACT: Photosystem II (PSII) is the only enzyme in nature that can catalyze the challenging catalytic photooxidation of H_2O into four protons, four electrons, and O_2. Slowing down turnover of the O_2-evolving complex (OEC) is a plausible approach to gain mechanistic information on the reaction. However, modulating the kinetics of the reaction without perturbing the active site is a challenge. In this study, it is shown that the steady-state activity of cyanobacterial PSII is inhibited by small zwitterions, such as glycine betaine and β-alanine. We show that the binding of zwitterions is nondenaturing, is highly reversible, and results in the decrease of the rate of catalytic turnover by ~50% in the presence of excess zwitterion. Control measurements of photoinduced electron transfer in O_2-inactive PSII show that the inhibition by zwitterions is the result of a specific decrease in the rate of catalytic turnover of the OEC. Recovery of activity upon addition of an exogenous proton carrier (HCO_3^-) provides evidence that proton-transfer pathways, thought to be essential for the relay of protons from the OEC to the lumen, are affected. Interestingly, no inhibition is observed for spinach PSII, suggesting that zwitterions act specifically by binding to the extrinsic proteins on the lumenal side of PSII, which differ significantly between plants and cyanobacteria, to slow proton transfer on the electron donor side of PSII.

Photosystem II (PSII)[1] is the only enzyme in nature that catalyzes the light-triggered oxidation of water into O_2, four protons, and four electrons (*1*−*3*), a reaction that remains a challenge for artificial systems (*4*). PSII is present in all oxygenic photosynthetic organisms, and its catalytically active core has no known variation. Catalysis takes place in the O_2-evolving complex (OEC) (Figure 1), made up of four high-valent Mn and one Ca^{2+} that are linked by μ-oxo bridges, a redox-active tyrosine (Tyr_Z), and other protein residues that together function as a scaffold. In addition, the presence of a catalytic base is suggested for the thermodynamically efficient oxidation of water (*5*, *6*).

The OEC cycles through four light-driven one-electron oxidations, from S_0 (the most reduced state) to S_4 (the most oxidized state) (Scheme 1), to accumulate enough potential to oxidize water. O_2 is formed in the $S_3 \rightarrow [S_4] \rightarrow S_0$ transition, where $[S_4]$ is a transient and highly reactive intermediate. The presence of two exchangeable substrate water molecules in the S_3 state, as evidenced by mass spectrometry (*7*), indicates that the O−O bond is formed from two bound substrates. All of the S-state transitions involve electron transfer. In addition, extraction and transport of four protons are required for each full turnover of the S-state cycle. Due to a positive charge buildup in the latter stages of catalysis (*8*), proton transfers are likely to be coupled to electron transfer, either in a concerted or in a sequential fashion (*9*).

The cofactors responsible for electron transfer away from the OEC are well established (ref *3* and references cited therein). However, the specific route of proton transfer from the OEC to the lumen, also a requirement for catalytic turnover, is unclear. Following from the X-ray crystal structures (*10*, *11*), specialized channels within the protein scaffold have been invoked to carry out proton transfer, as well as to facilitate H_2O entry into and O_2 exit from the OEC (*12*−*17*).

Elucidating the mechanism of photooxidation of H_2O by PSII, especially the chemistry of highly reactive intermediates like $[S_4]$, has potential applications for the design of synthetic water-splitting processes. In PSII, strategies like perturbing the active site by single-point mutations (*18*, *19*) and metal ion substitutions (*20*, *21*) are employed to investigate the mechanism. An interesting approach would be to slow down the catalysis by using discrete, exogenous small molecules that selectively interact with the protein scaffold. This way, the active site remains unaffected, yet the catalysis could be significantly modulated. Such an approach not only would provide tools for mechanistic investigations but also could enable new avenues to investigate how the protein scaffold plays an active role in catalysis. The present study suggests a new and simple strategy for achieving this goal.

Glycine betaine (also referred to as "betaine"), sucrose, β-alanine, and glucosylglycerol are only a few of the many natural compounds that are synthesized within cells under osmolytic stress, referred to as osmolytes. Osmolytes are known to increase the T_m for denaturation of proteins (*22*), protect cells against severe salinity (*23*), and stabilize their native conformation (*24*, *25*). Glycine betaine is the natural osmolyte in many photosynthetic organisms, including certain plants (*26*) and cyanobacteria, especially in those living in high salinity (up to 200%) environments (*27*). The stabilizing effect of glycine betaine and sucrose on PSII has previously been shown (*28*), and they are both used as stabilizing agents for purified PSII preparations.

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[1]Abbreviations: Chl, chlorophyll; DCBQ, 2,5-dichlorobenzoquinone; DCPIP, 2,6-dichlorophenolindophenol; β-DM, β-dodecylmaltoside; DPC, diphenyl carbazide; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, O_2-evolving complex; PIPBS, 1,4-bis(4-sulfobutyl)piperazine; PSII, photosystem II; RDS, rate-determining step.

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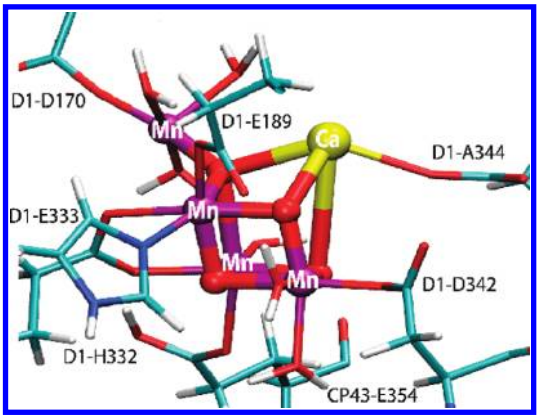
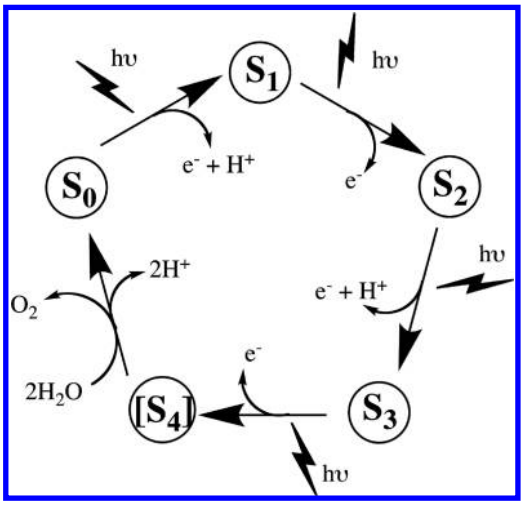


FIGURE 1: O_2-evolving complex (OEC). Reproduced from ref *49*.

Scheme 1: S-State Cycle



Herein, we report the kinetic modulation and the resulting decrease of catalytic rate (by ~50%) of steady-state activity of cyanobacterial PSII through favorable binding of a small zwitterion, glycine betaine. The successful recovery of activity by an exogenous proton carrier (HCO_3^-) provides evidence that proton-transfer pathways, thought to be essential for the relay of protons from the OEC to the lumen, are affected. We further discuss the nature and possible sites of this zwitterion-triggered interaction and provide evidence for its species dependence.

EXPERIMENTAL PROCEDURES

Purification of PSII. PSII membrane fragments were isolated from fresh organic spinach using the protocol developed by Berthold et al. (*29*), and spinach PSII core complexes were purified using the method in ref *30* with some modifications. The hexahistidine tag on His-tagged CP47 *Synechocystis* PCC 6803 was engineered (*31*), and cells were grown (*32*) as described before. The metal-affinity purification procedure in Lakshmi et al. (*33*) was followed, except for the following adaptations: (1) All buffers contained 1.2 M glycine betaine (anhydrous; USB Corp.) and 10% (v/v) glycerol (ultrapure, MB grade; USB Corp.), instead of 25% (w/v) glycerol. (2) The ultracentrifuge step in Tang and Diner (*34*) was employed after the cell breakage was completed, and the pellet was suspended in a different buffer, buffer A (50 mM MES−NaOH, pH 6.0, 1.2 M glycine betaine, 20 mM $CaCl_2$, 5 mM $MgCl_2$, and 10% (v/v) glycerol), to a minimal volume so that [Chl] = 1.1 mg/mL. (3) After thylakoid membrane extraction and isolation of the solubilized material from the debris, the solubilized PSII extract was directly loaded onto Ni^{2+}-NTA agarose (QIAgen), preequilibrated with buffer B (50 mM MES−NaOH, pH 6.0, 1.2 M glycine betaine, 20 mM $CaCl_2$, 5 mM $MgCl_2$, 10% (v/v) glycerol, and 0.03% β-DM (Anatrace)). No imidazole was added in this step. Instead of mixing and incubating, the loaded crude extract was washed immediately with 160 mL (4 bed volumes) of buffer B. Elution was carried out with 160 mL of buffer B plus 250 mM imidazole (Sigma-Aldrich). (4) The eluate plus 1 mM EDTA was concentrated in Centricon centrifugal filter devices with a 100 kDa cutoff (Millipore), prerinsed with buffer B, for ~2 h at 3500*g* in a Sorvall HS-4 swinging bucket rotor to a total volume of <5 mL. The solution was then desalted on a Bio-Rad G-25 column (preequilibrated with buffer B).

Measurement of Steady-State O_2-Evolving Activity. O_2 evolution was monitored by a Clark-type electrode, within a chamber kept at 25 °C by a temperature controller, and continuously stirred. Photochemistry was initiated by an Oriel 1000 W quartz tungsten halogen lamp, fitted with a distilled water-filled filter, a heat filter, and a 610 nm cutoff filter (LP 610). All buffers were incubated in the chamber for ~5 min before the PSII aliquot was introduced. Electron acceptors, 250 μM 2,5-DCBQ, and 1 mM $K_3Fe(CN)_6$ were added to all samples prior to the addition of the PSII aliquot. PSII samples were kept on ice and added to the chamber immediately before the assay. As the PSII aliquots were in the microliter range, only 1 min of incubation was necessary for temperature equilibration before measurement of the activity. Typical sample activities at pH optima were ~5000 μmol of O_2/(mg of Chl·h) for PSII core complexes isolated from *Synechocystis* PCC 6803 and ~400 μmol of O_2/(mg of Chl·h) for spinach PSII membranes. The first 30 s of the constant increase in the recorded O_2 evolution was used to calculate the activity.

PSII samples were suspended in buffer media with different pH values as described below. Adjustments of pH were made by addition of a dilute solution of NaOH, and the pH was monitored at room temperature by using a pH electrode (Fisher Thermo Scientific). The following buffers were used: glycine betaine/β-alanine buffer containing 1.2 M glycine betaine (anhydrous; USB Corp.) or 1.2 M β-alanine (Sigma-Aldrich), 20 mM MES, 20 mM 1,4-bis(4-sulfobutyl)piperazine (PIPBS; GFS Chemicals), 20 mM $CaCl_2$, 10 mM NaCl, and 30% (v/v) glycerol; sucrose buffer containing 1.0 M sucrose (J. T. Baker), 20 mM MES, 20 mM PIPBS, 25 mM $CaCl_2$, and 10 mM NaCl.

O_2-evolution activity was measured two to three separate times for each data point. The data were plotted against the pH of the solution that was measured after the assay, as in Figure 2.

Reversibility of Inhibition Measurements. Buffers with glycine betaine, sucrose, or β-alanine were prepared separately and adjusted to various pH values, ranging from pH 4 to pH 8. These will be called the "trial buffers". A constant O_2-evolving activity buffer was designated to be the "assay buffer" (1.0 M sucrose−NaOH, pH 6.5, 50 mM MES, 25 mM $CaCl_2$, 10 mM NaCl). Each trial buffer (50 μL) was incubated at 25 °C for 5 min to reach a stable temperature. The PSII sample (5 μL, usually in the 0.05−0.02 mg of Chl/mL range) was then pipetted into the trial buffer, thoroughly mixed, and incubated for 1 min at 25 °C in the dark (this is the same incubation time used for samples assayed as a function of pH with various concentrations of osmolyte). The incubation time was started after the sample and the buffer were properly mixed. Then, a 10 μL aliquot of the

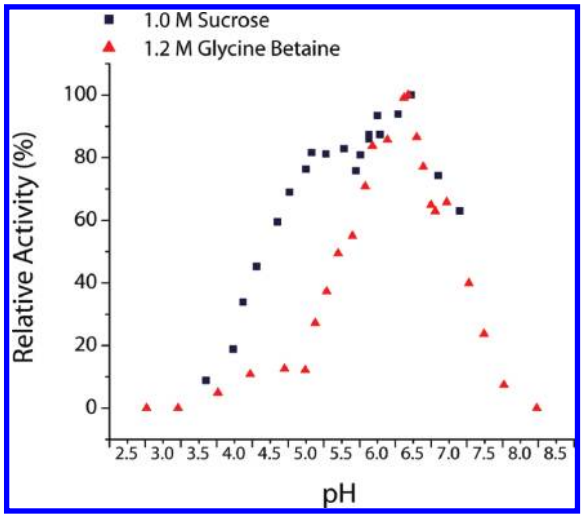


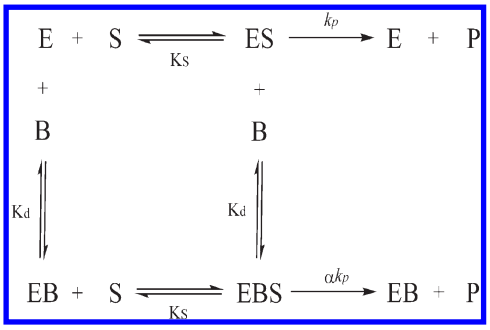
FIGURE 2: pH profiles of O_2-evolution activity of PSII core complexes isolated from *Synechocystis* PCC 6803. The assays were done in buffers containing either 1.0 M sucrose or 1.2 M glycine betaine. Each point is an average of at least two separate measurements. The highest rate measured for the series of assays done with each osmolyte was set to 100%, and the rest of the values were scaled accordingly.

incubated sample was introduced into the activity chamber (into the solution containing the assay buffer and electron acceptors), and an activity assay was run, as described above. For each trial buffer, two separate incubations and assays were conducted. The highest value of activity (trial buffer pH = 6.5) was taken as 100% for each osmolyte. All of the rest of the activities were then plotted relative to this value, as shown in the Supporting Information (Figure S-2).

Electron-Transfer Activity Assays. The molar extinction coefficient of 2,6-dichlorophenolindophenol (DCPIP) is pH and buffer dependent (*35*). Therefore, first, the isosbestic point of DCPIP was determined for two different buffer solutions: (1) 1.2 M glycine betaine, 30% (v/v) glycerol, 20 mM MES, 20 mM PIPBS, 20 mM $CaCl_2$, and 10 mM NaCl and (2) 1.0 M sucrose, 20 mM MES, 20 mM PIPBS, 20 mM $CaCl_2$, and 10 mM NaCl. These buffer solutions were titrated to pH values ranging from 4.0 to 7.5 using dilute NaOH. Spectra of 35 μM DCPIP in 1 mL of buffer solution were recorded as a function of pH. An isosbestic point of 533 nm was obtained for buffer condition 1 and 530 nm for buffer condition 2. Extinction coefficients were obtained by plotting the increase in absorption at the isosbestic points as a function of [DCPIP] (0.01, 0.02, 0.045, 0.06, 0.1 mM), with the pH of each buffer set to 5.0.

The electron-transfer assays were conducted with O_2-inactive PSII isolated from *Synechocystis* PCC 6803 that was incubated at 0 °C for ~4 h prior to the experiment to inactivate the OEC. The activity of the sample at the end of the incubation was ~200 μmol of O_2/(mg of Chl·h). The electron-transfer assay solutions included the O_2-inactive PSII at a concentration of 10 μg of Chl/mL, 35 μM DCPIP (dissolved in water), 1 mM diphenyl carbazide (DPC) (dissolved in DMSO), and either buffer 1 or 2, added to a total volume of 1.0 mL. The residual electron-transfer activity from active PSII was corrected for by control experiments that were conducted without DPC. The change in absorbance was recorded at the designated isosbestic points as a function of time, upon illumination with an EIKO EKE (150 W, 21 V) MR16 halogen lamp fitted with a water filter. Each scan was 3 min long. The results were recorded with a Cary 50 spectrophotometer, by using the Cary WinUV kinetics application.

Scheme 2: Modeled Glycine Betaine Binding to PSII[a]



[a]The model describes the effect on the catalytic activity by a factor α (where 0 < α < 1) when betaine is bound (E = PSII, S = substrate (water), B = betaine, P = product, K_s = substrate dissociation constant, K_d = betaine dissociation constant, and k_p = catalytic step(s)).

K_d Measurements. O_2-evolving activities were measured for samples as a function of betaine concentration at a constant pH of 5.10 (pH values of aliquots were measured after their activity assay was completed). Betaine concentrations used were 1 μM, 1 mM, 10 mM, 100 mM, 250 mM, 500 mM, 750 mM, 1.0 M, and 1.2 M. PSII samples used in the data collection were previously washed with a buffer that did not contain glycine betaine (buffer B minus 1.2 M glycine betaine), so that the concentration of betaine in the medium could be controlled with accuracy. The resulting data (average of three separate measurements) were fitted with a rate equation (eq 1) that was derived from the binding model shown in Scheme 2 (see derivation in Supporting Information). The simplified formula is given below. This simplification assumes the dissociation constant for water, K_s, to be $\ll$ [H_2O]; therefore, any term that includes (K_s/[H_2O]) approaches zero and can be neglected. $V_{max_{app}}$ is the observed rate of O_2 evolution, V_{max} is the theoretical maximum, [B] is the betaine concentration, K_d is the apparent betaine dissociation constant, and α is the factor by which the catalytic rate is affected.

$$V_{max_{app}} = \frac{V_{max}\left(1+\frac{\alpha[B]}{K_d}\right)}{1+\frac{[B]}{K_d}} \quad (1)$$

Chemical Rescue of Activity by Bicarbonate. Chemical rescue of activity experiments were done using buffers containing either (1) 1.2 M glycine betaine, 30% (v/v) glycerol, 200 mM MES−NaOH, pH 5.8, 20 mM $CaCl_2$, and 10 mM NaCl or (2) 1.0 M sucrose, 200 mM MES−NaOH, pH 5.8, 20 mM $CaCl_2$, and 10 mM NaCl. To these buffers were added aliquots from a 1 M $NaHCO_3$ stock solution to a final concentration of 0, 5.6, 8.4, 11.2, or 22.4 mM, respectively, ~5 min before the activity assay was run. In order to keep the ionic strength of the buffer solutions constant, NaCl (from a 1 M stock) was added to compensate for the differences in $NaHCO_3$ concentration.

The activity assays were run as described above, but using white light, and each measurement was repeated at least three separate times. The pH of each solution in the assay chamber was measured after the activity assay to confirm that there was no significant change of pH during the experiment.

RESULTS

pH Dependence of Activity. When PSII isolated from *Synechocystis* PCC 6803 was assayed in any of the buffers used,

Table 1: Effective pK_a Values Obtained from Fits to the Respective Profiles of O_2-Evolution Activity vs pH

	pK_{a1}	pK_{a2}
cyanobacterial PSII in 1.2 M glycine betaine	6.13 ± 0.09	6.95 ± 0.09
cyanobacterial PSII in 1.0 M sucrose	4.64 ± 0.06	7.0 ± 0.5
cyanobacterial PSII in 1.2 M β-alanine	5.68 ± 0.08	6.9 ± 0.2
untreated plant PSII[a]	5.20 ± 0.04	6.83 ± 0.05
Sr^{2+}-substituted plant PSII[a]	6.2 ± 0.2	6.7 ± 0.2
plant PSII in 1.2 M glycine betaine	4.76 ± 0.07	7.57 ± 0.09
plant PSII in 1.0 M sucrose	4.8 ± 0.1	7.7 ± 0.2

[a]From ref *36*.

the pH dependence of its steady-state activity could be fitted into a diprotic model, with effective pK_a's near 4.6 (pK_{a1}), and 7.0 (pK_{a2}), calculated as described previously (*36*). While cyanobacterial PSII assayed in buffers containing osmolytes shows practically no change in pK_{a2}, an ~1.5 pH unit increment is observed for pK_{a1} upon changing the osmolyte from the nonionizable molecule sucrose to the zwitterionic molecules betaine and β-alanine (Table 1, Figure 2, and Supporting Information Figure S-1).

Measurements of the reversibility of all three treatments reveal that > 80% of the PSII activity could be recovered by diluting the osmolyte-containing sample into pH 6.5 buffer over the pH range from 4 to 8 (Supporting Information Figure S-2). Electron-transfer activity assays of O_2-inactive PSII (Figure 3) show no effect of betaine on electron transport from DPC to DCPIP. There is a pH dependence for the electron-transfer assay, showing a decrease of activity at pH > 5.5 (Figure 3). However, this pH dependence does not agree with the pH dependence of steady-state O_2-evolution activity (Figure 2), indicating that the modulation of O_2-evolution activity by betaine below pH 6 is due to inhibition of the OEC turnover.

Concentration Dependence of Betaine on Steady-State PSII Activity. At 1 μM and 1 mM betaine concentrations, the pH profile of the steady-state activity matched well to that in 1.0 M sucrose buffer (Supporting Information Figure S-3). However, steady-state activity was found to decrease as a function of increasing betaine concentration above 1 mM. When concentrations in the nanomolar range were used, there was some loss of O_2-evolution activity, probably due to destabilization of PSII in our buffer conditions. Therefore, these data were not included in any further studies. An *apparent* K_d value could be obtained from the decrease of steady-state O_2-evolving activity by comparing relative activities of samples with varied betaine concentrations ranging from 1 μM to 1.2 M at constant pH (Figure 4). The relative activity vs [betaine] gives approximately a hyperbolic plot, consistent with *partial noncompetitive inhibition* (*37*), shown in Scheme 2. In this model, betaine can bind to both the free enzyme (E) and the enzyme−substrate (ES) complex with the same affinity and without perturbing substrate binding or completely blocking the activity. However, compared to unbound ES, betaine binding is predicted to affect the reaction rate by a factor, α, where $\alpha > 0$ if the interaction is not completely inhibitory and $\alpha < 1$ if the catalytic rate is decreased. A rate equation was derived (eq 1) according to this model (complete derivation in Supporting Information) and was fitted to the experimental data, yielding $K_d = 0.33 \pm 0.07$ M, the theoretical maximum of relative activity, $V_{max} = 98.9 \pm 1.6\%$, and the α-factor = 0.45 ± 0.04 (Table 2).

Chemical Rescue of Activity by Bicarbonate. In order to test whether the inhibition of O_2-evolution activity induced by

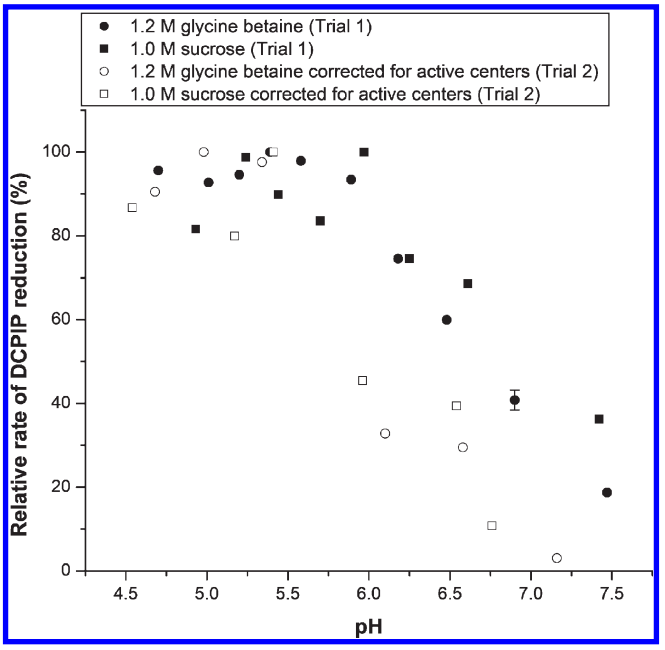


FIGURE 3: Electron-transfer activity assays, showing relative rates of DCPIP reduction as a function of pH, monitored as the decrease of the isosbestic points of DCPIP in betaine-containing buffer (533 nm) and sucrose-containing buffer (530 nm). Data sets labeled "trial 1" were conducted with PSII samples that were incubated on ice for a duration of ~4 h, resulting in significant Mn depletion. Data sets labeled "trial 2" were run with similarly treated PSII samples but are corrected for the active PSII centers present. (Raw data showing DCPIP reduction with and without DPC can be found in Supporting Information.) An average of two separate measurements is plotted for each data set in trial 2, and an average of multiple measurements is plotted for each data set in trial 1. A representative error for three separate measurements is shown in the plot. The resulting plot shows no apparent pH dependence in the electron-transfer pathway below pH 5.5.

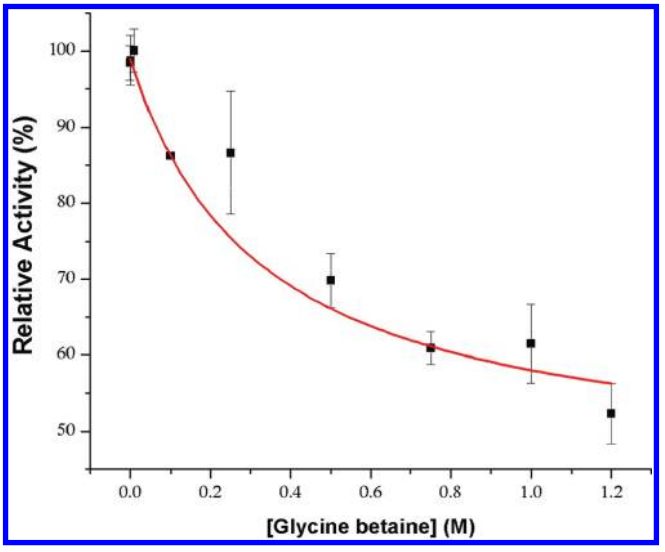


FIGURE 4: The effect of betaine concentration on O_2-evolution activity at constant pH (pH = 5.10). Each point is an average of three measurements. The least-squares fit, based on the binding model (Scheme 2), is calculated from eq 1 (the derivation is shown in the Supporting Information).

betaine is due to blockage of proton-transfer pathways on the electron-donor side of PSII, a chemical rescue experiment was performed using bicarbonate. A progressive recovery of the activity was observed when increasing concentrations of bicarbonate

Table 2: Results of the Fit in Figure 4

χ_2	0.89918
R^2	0.98323
K_d (M)	0.33 ± 0.07
V_{max} (%)	98.9 ± 1.6
α	0.45 ± 0.04

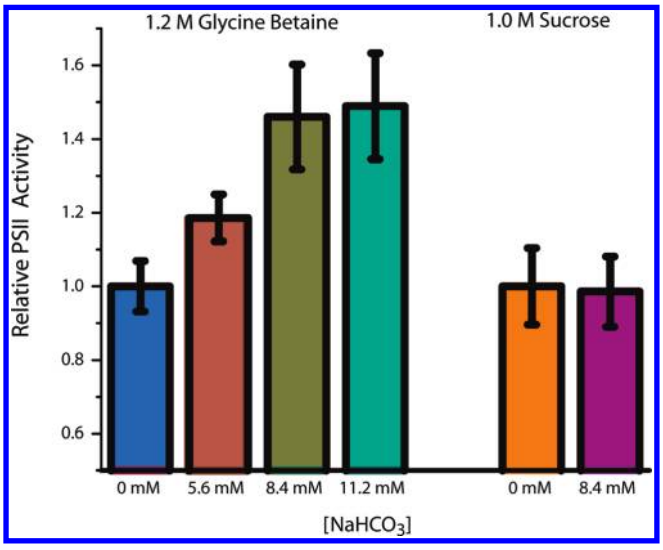


FIGURE 5: Chemical rescue of steady-state O_2-evolution activity with an exogenous proton carrier, HCO_3^-. Concentrations on the *x*-axis show the amount of $NaHCO_3$ that was added to the assay solution from a stock solution. Sucrose-containing buffer was used as a control, demonstrating that additional HCO_3^- does not have a significant effect on activity of PSII suspended in sucrose-containing buffer. The activity of samples with no HCO_3^- added was taken as unity for each buffer, and the rest of the values were scaled accordingly. 100% corresponds to the activities at pH 5.8 for PSII in buffer containing each osmolyte, as shown in Figure 2.

were added to *Synechocystis* PSII at pH 5.8 in the presence of 1.2 M betaine. The activity increased by ~50% when 8.4 mM $NaHCO_3$ was added to PSII suspended in the betaine-containing buffer (Figure 5). However, addition of this same concentration of bicarbonate had no effect on the O_2-evolution activity of *Synechocystis* PSII in the presence of sucrose. Measurements with concentrations higher than ~12 mM $NaHCO_3$ gave an indication that even more of the activity can be recovered from *Synechocystis* PSII at pH 5.8 in the presence of 1.2 M betaine. However, rapid CO_2 evolution was observed with these high concentrations of bicarbonate, which interferes with the O_2-detecting Clark electrode, rendering the quantification of the activity unreliable; hence, these measurements have been omitted.

pH Dependence Differences between Cyanobacterial and Higher Plant PSII. The significant change in pK_{a1} of cyanobacterial PSII brought about by betaine was not observed for PSII isolated from spinach (Table 1 and Supporting Information Figure S-4), consistent with previous work (*38*). The calculated pK_{a1} was 4.76 ± 0.07 in 1.2 M betaine-containing buffer and 4.8 ± 0.1 in 1.0 M sucrose-containing buffer, while pK_{a2} was 7.57 ± 0.09 in betaine-containing buffer and 7.7 ± 0.2 in sucrose-containing buffer.

DISCUSSION

Previously, the pH dependence of both steady-state PSII activity (*36*) and specific S-state transitions (*39*) has been investigated in order to characterize proton-transfer processes associated with turnover of the OEC. In this study, the pH dependence of steady-state O_2-evolution activity has been measured to investigate the effects of osmolytes on proton-transfer processes associated with turnover of PSII. Our results show a pronounced effect of the nondenaturing zwitterions glycine betaine and β-alanine on the steady-state O_2-evolving activity of PSII from *Synechocystis* PCC 6803 at pH values below the optimal pH for activity of 6.5, indicating that a rate-limiting deprotonation or proton-transfer event is being modulated by the binding of these zwitterions to PSII. This zwitterionic effect was observed for cyanobacterial PSII but was not observed for higher plant PSII. In this section, we discuss the nature, site, and effects of zwitterion binding in cyanobacterial PSII.

Modulation of Activity Is Zwitterion Dependent. Glycine betaine, a small zwitterion, is shown to cause approximately a 50% decrease in the rate of O_2 evolution at pH = 5.1 (Figure 4, Table 2). The pH profile of cyanobacterial PSII activity in the presence of betaine is distinctly different from that in sucrose-containing buffer, with a shift of pK_{a1} to higher pH by 1.5 units (Table 1). The concentration dependence for inhibition fits to a partial noncompetitive inhibition model (Scheme 2) (*37*) with weak binding of betaine to cyanobacterial PSII (K_d = 0.33 ± 0.07 M), where the O_2-evolving activity of PSII is significantly slowed upon betaine binding. At low concentrations of betaine ([betaine] ≤ 1 mM) and in the absence of sucrose, the pH profile of cyanobacterial PSII activity matches that of PSII assayed in sucrose-containing buffer, indicating that sucrose does not interact with PSII in a way that affects the steady-state activity. Thus, use of sucrose as a stabilizing agent provides a good control for our experiments. The shift in pK_{a1} induced by betaine is similar to the ΔpK_a = 1 observed in the presence of β-alanine, another small zwitterion. These results demonstrate that small zwitterions are required to bring about the observed significant decrease in PSII activity below pH 6.5.

pH Dependence of Activity. Previous pH dependence studies of steady-state activity of Sr^{2+}-substituted PSII (*36*) revealed a significant shift of pK_{a1} (Table 1), suggesting that pK_{a1} is associated with turnover of the OEC. Our electron-transfer activity assays (Figure 3), which show that the electron-transfer pathway in O_2-inactive PSII is not pH dependent over the pH range from 4.7 to 5.5 and decreases as pH is raised, indicate that pK_{a1} is directly related to a protonation reaction associated with the OEC. Our results agree with previous work showing that S-state transitions (*39*) and proton release that accompanies these transitions (*40*) can be rate limiting at low pH, whereas, *in vivo*, the rate-determining step (RDS) for O_2 evolution is the second reduction of the membrane-soluble quinone (Q_B) (0.7−0.8 ms) and its diffusion into the bulk solution (2−3 ms) (*41*). Moreover, our fitted pK_{a1} for O_2-evolving activity of PSII in sucrose, 4.64 ± 0.06, agrees with the pK_a of ~4.8 determined from studies of the pH dependence for S-state turnover (*39*), consistent with the hypothesis that pK_{a1} is related to a proton-transfer process in the OEC. Taken altogether, these findings indicate that, in the acidic pH regime where pK_{a1} is observed, the RDS for O_2 evolution from purified PSII is associated with a proton-transfer reaction in the OEC and that the rate of this reaction is modulated by betaine. Further evidence for an effect of betaine on the proton-transfer reactions associated with turnover of the OEC comes from our observation that inhibition by betaine can be reversed by the addition of an exogenous proton-transfer agent: HCO_3^- (Figure 5).

Binding of Betaine to PSII. Conformational changes in the PSII protein scaffold have been observed for each S-state transition by Fourier-transform infrared (FTIR) spectroscopy (*42*, *43*). Glycine betaine's possible interactions with the peptide backbone, favoring amide N and excluding the amide carbonyl O (*44*), may play a role in stabilizing some conformations of the protein scaffold and, thereby, hindering the conformational changes that are required for catalysis to occur.

It is also possible that zwitterions such as betaine may bind to residues that are important for proton transfer from the OEC to the lumen. Deprotonation reactions and the relay of protons away from the OEC were suggested to have a remarkable impact on the thermodynamics of water oxidation (reviewed in ref *45*). Any restraint on proton transfer is, thus, expected to significantly disfavor water oxidation. The observation that the zwitterion-mediated inhibition of PSII activity can be recovered upon introduction of an exogenous proton-transfer agent, HCO_3^- (Figure 5), supports this possibility. As a small weak acid, HCO_3^- has previously been shown to rescue the rate of proton transfer associated with reduction of Q_B in bacterial reaction centers with impaired proton-transfer pathways (*46*). Known to be excluded from direct involvement in catalytic water oxidation (*47*−*49*), HCO_3^- also allowed us to uncouple proton-transport processes from deprotonation events associated directly with the OEC and selectively elucidate the former. The partial recovery (≥50% at pH 5.80) of steady-state PSII activity by HCO_3^-, thus, provides strong evidence for the impairment in the necessary proton-transport processes associated with turnover of the OEC.

Nature and Site of Zwitterionic Interactions. Previous studies on compounds that associate with the OEC revealed that the Mn cluster has high steric selectivity, favoring the small Lewis base, NH_3, which is considered analogous to substrate water (*50*, *51*). Betaine, a quaternary amine, is larger in size than amines capable of binding in proximity to the OEC, and therefore, its direct interaction with the OEC can be ruled out. Instead, it is likely that betaine interacts favorably with the protein scaffold of PSII, by binding to either the peptide backbone, ionizable amino acid side chains, or aromatic residues.

The existence of channels that allow for controlled entry of H_2O into and release of protons and O_2 from the OEC has long been a working hypothesis (*12*−*17*, *52*−*54*). Most evidence for these channels has been derived from identification of suitable pathways directly from X-ray crystal structures (*12*, *15*, *53*) and by use of computational methods (*14*, *16*, *17*, *52*, *54*). There is a consensus on the existence of proton-exit pathways, in particular, as protons are released against a proton gradient within the thylakoid membranes *in vivo*. These pathways are thought to start from the OEC and possibly branch out and end in the lumen and to be composed of a series of aligned ionized amino acid residues with fine-tuned pK_a values (*14*).

Favorable interactions between betaine and ionizable residues in the proton-exit pathways are expected to obstruct proton release into the bulk solvent and cause a change in the electrostatic environment in the OEC. As release of four H^+ is required for catalytic turnover, a significant modulation of k_{cat} is expected if proton-transfer pathways are inhibited. Indeed, an ∼50% decrease in the k_{cat} (at pH 5.10) was observed in the presence of excess zwitterion. This effect is not rate determining when pH > 6.5, where proton transport and release would be more favorable, and is also reversed by the presence of exogenous proton carriers, like HCO_3^-. Recent work conducted by Gabdulkhakov et al. (*12*) supports the possibility that betaine could suitably fit into channels within PSII, while that by Capp et al. (*44*) demonstrates that betaine can bind favorably to ionized and aromatic amino acid residues, as well as amide nitrogens in these channels.

In light of our measurements and previous work, we suggest that the interaction of glycine betaine with ionized amino acid residues in proton-transfer pathways is responsible for the observed decrease in PSII activity. The catalytic turnover is never completely blocked, however, due to the highly reversible nature of these interactions.

Species Dependence of Kinetic Modulation of Activity. Another experimental observation that needs to be addressed is the difference between cyanobacterial and higher plant PSII. Higher plant (spinach) PSII exhibits no change in its pH-dependent steady-state activity upon addition of betaine to the assay buffer (Supporting Information Figure S-4a, consistent with the results in ref *38*). Higher plant "PSII core complexes", believed to be better structural analogues of isolated cyanobacterial PSII, also failed to demonstrate any change of the pH dependence of activity in the presence of betaine (Supporting Information Figure S-4b). This difference might be attributed to an effect resulting from differences in the protein structure. A number of species-dependent structural differences are known based on genomic homologies (*55*): (i) the extrinsic polypeptides, which comprise the lumenal side of PSII and serve to seclude the OEC from the bulk environment, are known to be analogous, yet different in size and maybe in structure, and (ii) some of the extrinsic proteins, such as cytochrome c_{550}, are present in cyanobacteria, yet absent in higher plants. Even though these species-dependent differences in PSII structure, especially in the extrinsic polypeptides, have been known, their direct impact on catalytic function is not clear. Our investigation showing a distinct difference in the effect of betaine on the O_2-evolving activity of PSII from different species is, thus, a new observation that intrinsic differences in protein structure significantly modulate the function of PSII.

The absence of kinetic modulation of activity in spinach PSII by betaine implies that the structure of plant PSII is robust enough to retain its catalytically active form despite high concentrations of zwitterions. It is also conceivable that plant PSII includes a more diverse and fluid set of pathways to transport and release protons, which would render the binding of betaine ineffective and prevent inhibition of proton-transfer events upon binding of betaine to PSII from higher plants.

In summary, our studies show that small zwitterions slow the rate of proton transfer away from the OEC significantly through low affinity and reversible binding to the PSII protein scaffold. Through this zwitterionic modulation, we demonstrate a method to make the proton-transfer-dependent processes in the OEC turnover slower than the RDS of the overall PSII turnover. This novel mode of modulation could be a promising approach in future attempts to elucidate and maybe isolate the transient [S_4] state, whose formation and decay require proton transfer. We also demonstrate new experimental evidence for the existence of preestablished proton-transfer channels in PSII, possibly aligned with amino acid residues with fine-tuned pK_a values. Our findings also suggest that species-dependent variations in PSII structure directly modulate the catalytic function, a phenomenon that has not previously been shown experimentally for PSII. Finally, we report the first use of exogenous, nondenaturing zwitterions as selective inhibitors of proton-transport processes. This newly

uncovered effect could be used for mechanistic studies of other proton-transfer proteins.

SUPPORTING INFORMATION AVAILABLE

The pH dependence of activity in 1.2 M β-alanine, reversibility studies, concentration dependence of betaine, inhibition studies of spinach PSII membranes and spinach PSII core complexes, relative rates of DCPIP reduction with and without DPC, and derivation of the rate equation for partial noncompetitive inhibition. This material is available free of charge via the Internet at http://pubs.acs.org.

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