Sucrose and Glycerol Effects on Photosystem II

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ABSTRACT Photosystem II catalyzes the oxidation of water and the reduction of plastoquinone. The active site cycles among five oxidation states, which are called the S_n states. PSII purification procedures include the use of the cosolvents, sucrose and/or glycerol, to stabilize water splitting activity and for cryoprotection. In this study, the effects of sucrose and glycerol on PSII were investigated. Sucrose addition was observed to stimulate the steady-state rate of oxygen evolution in the range from 0 to 1.35 M. Glycerol addition was observed to stimulate oxygen evolution in the range from 0 to 30%. Both cosolvents were observed to be inhibitory at higher concentrations. Sucrose addition was shown to have no effect on the rate of Q_A^- oxidation or on the K_M for exogenous acceptor. PSII was then treated to remove extrinsic proteins. In these samples, sucrose addition stimulated activity, but glycerol addition was inhibitory at concentrations higher than ~0.5 M. This inhibitory effect of glycerol at relatively low concentrations is attributed to glycerol binding to the active site, when extrinsic subunits are not present. Reaction induced FTIR spectra, associated with the S_1 to S_2 transition of the water-oxidizing complex, exhibited significant differences throughout the 1,800–1,200 cm^{-1} region, when glycerol- and sucrose-containing samples were compared. These measurements suggest a cosolvent-induced shift in the pK_A of an aspartic or glutamic acid side chain, as well as structural changes at the active site. These structural alterations are attributed to a change in preferential hydration of the oxygen-evolving complex.

INTRODUCTION

In the photosynthetic electron transport chain, photosytem II utilizes light energy to catalyze the oxidation of water to produce molecular oxygen. PSII is a multisubunit enzyme that is located in the thylakoid membrane. Production of molecular oxygen occurs through a series of four oxidation reactions, which are accumulated on a tetranuclear Mn cluster. The sequentially oxidized forms of the catalytic site are known as the S_n states (Joliot and Kok, 1975). Electron transfer in PSII is initiated by photoexcitation of the primary chl donor, P_{680}. P_{680}* reduces a pheophytin molecule, which in turn reduces a plastoquinone acceptor molecule, Q_A. Q_A^- transfers the electron to a second plastoquinone acceptor, Q_B. Although Q_A is a single electron acceptor, the terminal electron acceptor, Q_B, acts as a two proton/two electron acceptor. The charge-separated state, $P_{680}^+Pheo^-$, is stabilized against charge recombination by the fast oxidation of $Pheo^-$ and by the reduction of P_{680}^+ through a tyrosine residue, Z. Z• is reduced by the Mn cluster during each S-state transition. Tyrosine D forms a stable radical, D• (reviewed in Britt, 1996).

Higher plants and cyanobacteria require at least six intrinsic proteins for oxygen evolution (Barry et al., 1994), plus the inorganic cofactors, Ca^{2+} and Cl^-. Plant PSII contains three extrinsic polypeptides, known as the 18, 24, and 33 kDa (or manganese stabilizing protein, MSP). In the absence of the 18- and 24-kDa proteins, high concentrations of calcium and chloride are required for maximal oxygen evolution activity (Ghanotakis et al., 1984a; Miyao and Murata, 1984, 1985). Exogenous reductants have increased access to the active site under these conditions (Ghanotakis et al., 1984b; Tamura and Cheniae, 1985). EPR studies have shown that the 18- and 24-kDa proteins modulate the magnetic properties of the Mn cluster (Campbell et al., 1998; de Paula et al., 1986). In the absence of all three extrinsic subunits, the steady-state oxygen evolution rate decreases, the Mn cluster is destabilized, high concentrations of Cl^- are required for stability, and the rate of the S-state transitions are slowed (reviewed in Seidler, 1996).

The mechanism by which PSII oxidizes water has not as yet been elucidated (for reviews, see Haumann and Junge, 1999; Messinger, 2000; Vrettos et al., 2001; Yocum and Pecoraro, 1999). One difficulty in studying the mechanism of oxygen evolution by standard biochemical techniques is that the substrate is the solvent. To investigate the dependence of activity on substrate binding, two approaches have been used. In the first, isotopically labeled water was used to determine the rate of substrate exchange as a function of flash number (for example, see Messinger, 2000, and references therein). In the second, cosolvent/water mixtures have been used to study PSII kinetics. Sucrose and/or glycerol have been used in many PSII purification procedures as cryoprotectants and to improve PSII stability (for examples, see Berthold et al., 1981; Bricker et al., 1998; Kirilovsky et al., 1992; MacDonald and Barry, 1992; Noren et al., 1991). For example, glycerol has been shown to stabilize oxygen evolution and maintain binding of a 9-kDa PSII subunit in the cyanobacterium, *Phormidium laminosum* (Stewart et al., 1985). The addition of glycerol has been shown to alter PSII proton release stoichiometries, suggesting a cosolvent-induced effect on the pK_A values of ionic

Submitted February 16, 2003, and accepted for publication May 6, 2003.

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Abbreviations used: PSII, photosystem II; FTIR, Fourier transform infrared; chl, chlorophyll; EPR, electron paramagnetic resonance; MES, 2-(*N*-morpholino)ethanesulfonic acid; DCBQ, 2,6-dichloro-*p*-benzoquinone.

0006-3495/03/08/1317/09 $2.00

groups near the active site (Haumann et al., 1997). In intact thylakoids, but not in broken thylakoids, polyethylene glycol and sucrose were reported to increase the oxygen evolution rate (Ananev, 1988). Ethylene glycol concentrations above 50% have been shown to cause the loss of PSII extrinsic proteins and PSII oxygen evolving activity (Hillier et al., 1997). Taken together, this previous work implies that cosolvents can have several different effects on PSII.

In this article, the effects of sucrose and glycerol on intact, salt-washed, and urea-washed PSII are investigated. In intact PSII, the effect of cosolvent addition is biphasic, with a stimulatory and an inhibitory concentration range. In PSII, which lacks the extrinsic proteins, glycerol is inhibitory even at low concentrations, whereas the effects of sucrose are similar to the sucrose effects observed in intact PSII. FTIR spectroscopic studies provide evidence that there is a conformational change at the catalytic site, when glycerol and sucrose containing samples are compared. These spectroscopic alterations are consistent with a change in hydration of the active site.

MATERIALS AND METHODS

Photosystem II preparations

Intact PSII was isolated from market spinach (Berthold et al., 1981). Salt-washed PSII (1 mg chl/ml) was treated in a 1:1 v/v ratio with 4 M NaCl, shaken on ice for 30 min in the dark, and centrifuged at 20,000 rpm for 30 min. This procedure removes the 18- and 24-kDa extrinsic proteins (Ghanotakis et al., 1984a). The pellet was resuspended in SMN buffer containing 10 mM NaCl, 50 mM MES-(NaOH) pH 6.0, and 0.4 M sucrose. Urea-washed PSII was generated by treatment of salt-washed PSII with a urea buffer, containing 240 mM NaCl, 50 mM MES-(NaOH) pH 6.0, 0.4 M sucrose, and 3.1 M urea. Urea treatment has been shown to remove the 33-kDa protein while preserving the Mn cluster (Bricker, 1992; Miyao and Murata, 1983b). Urea buffer was mixed with salt-washed PSII (1 mg chl/ml) at a 5:1 v/v ratio. The mixture was shaken on ice in the dark for 30 min and then centrifuged at 20,000 rpm for 15 min. The pellet was homogenized in a buffer containing 0.4 M sucrose, 50 mM MES-(NaOH) pH 6.0, and 200 mM NaCl and centrifuged at 20,000 rpm for 15 min. The chlorophyll concentration of each PSII preparation was determined as previously described (Barry, 1995; Lichtenthaler, 1987).

Oxygen evolution assays

Oxygen evolution assays were performed (Barry, 1995) using a Clark electrode (YSI 5300, YSI Inc., Yellow Springs, OH). Each assay contained 15–25 μg chl/ml, 530 μM recrystallized DCBQ, 10 mM NaCl, 50 mM MES-NaOH, pH 6.0, and varying concentrations of sucrose or glycerol. Recrystallized DCBQ was added from a concentrated solution in ethanol; the final ethanol concentration was $\leq$1%. The sucrose concentrations were 0, 0.40, 0.45, 0.65, 0.68, 1.35, 1.50, 1.65, and 1.80 M. The glycerol concentrations were 0, 15, 25, 30, 35, 40, 45, and 50% (v/v). The conversion from percent to molar was based on a glycerol density of 1.26 g/mol (CRC Handbook of Chemistry and Physics, 1972). The total volume for each assay was 1.5 ml. For salt-washed PSII, 10 mM $CaCl_2$ was added to the assay volume. For urea-washed PSII, 10 mM $CaCl_2$ and 100 mM NaCl were added to the assay buffer. After addition of all components, the mixture was stirred in the dark for ~15 s. Illumination was then provided by a Dolan Jenner fiberoptic illuminator, equipped with red and heat filters. The light intensity was ~2 mEinstein $(m^2\ s)^{-1}$. The electrode was standardized with deionized water, and the presence of cosolvent was observed to have no significant effect (<1%) on this standardization, given the error in the measurement (~2%). The oxygen evolution rate was calculated from data acquired between 10 and 20 s after the beginning of illumination. For each concentration of sucrose and glycerol, the steady-state oxygen evolution assay was performed six times, and the data were averaged. To measure the dependence of oxygen evolution rate on DCBQ concentration, the DCBQ concentrations were 0, 100, 250, 450, 530, 750, and 1,000 μM. The final ethanol concentration was constant and was 0.5%. Assays were performed four times, and the data were averaged.

Chl fluorescence

Chl fluorescence measurements were performed on a double-modulation fluorometer (FL-100, Photon Systems Instruments, Czech Republic), as previously described (Pujols-Ayala and Barry, 2002). Fluorescence measurements were performed with buffers containing 0, 0.4, 0.65, 1.35, and 1.8 M sucrose. The other components of the buffer were the same as those used for the oxygen evolution assays, except that either no DCBQ or 10 μM recrystallized DCBQ was used. Higher concentrations of DCBQ could not be used without quenching the fluorescence. The samples were stirred for 30 s and then dark adapted for 5 min. After dark adaptation, a preflash and a 5-min dark adaptation time were used to synchronize centers in the S_1 state. After the measurement of F_0, a single-turnover flash from the flash-lamp was provided, and the rate of fluorescence decay was measured. The flash measurements were repeated six times, at a repetition rate of 0.2 min^{-1}, and the data were averaged. The overall halftime of fluorescence decay was calculated by analysis with IGOR PRO software. Due to a flash-induced detector artifact, the first data point was 200 μs after the flash. Conditions were as follows: flash duration, 10 μs; measuring flash voltage, 50%; actinic flash voltage, 100%.

EPR spectroscopy

EPR samples were performed on salt-washed PSII, in buffers containing 50 mM MES-NaOH, pH 6.0, 15 mM NaCl, and 0.4 M sucrose, 1.35 M sucrose, 5% glycerol, or 30% glycerol. Recrystallized DCBQ (0.02 mM) was added as an exogenous electron acceptor from an ethanol solution; the ethanol concentration was $\leq$1%. The samples were concentrated to ~10 mg chl/ml by centrifugation and placed in a quartz EPR tube. Continuous, red-filtered illumination (5 min) was used to generate the S_2 EPR multiline signal at 200 K (de Paula et al., 1985; Zimmermann and Rutherford, 1984). Multiline spectra were collected at 10 K on a Bruker EMX spectrometer, equipped with an Oxford cryostat. Parameters were: 32-G modulation amplitude, 10-mW microwave power, 0.8-s time constant, 168-s scan time, 2,500-G sweep width, 8 total scans. Spectra were corrected for chlorophyll concentration through normalization to the number of tyrosyl D• spins present after 200-K illumination. Conditions for the D• EPR measurements were: 2-G modulation amplitude, 0.8-mW microwave power, 1.3-s time constant, 168-s scan time, 100-G sweep width, 4 total scans.

FTIR analysis

PSII samples at 3–4 mg chl/ml were mixed with 0.12 mM potassium ferricyanide and 0.02 mM recrystallized DCBQ, and pelleted at 20,000 rpm for 5 min. The low concentrations of exogenous acceptors, 0.12 mM potassium ferricyanide and 0.02 mM recrystallized DCBQ, were chosen on the basis of several criteria. First, samples showed rapid and reproducible oxidation of Q_A^-, as determined from fluorescence experiments (data not shown). Second, samples showed high retention of oxygen evolution activity after the measurement (70 $\pm$ 12%). Third, the DCBQ and ferricyanide concentrations were low enough that no significant spectral contribution was observed from acceptor-based redox reactions. For

spinach, higher concentrations of potassium ferricyanide, (20 mM), as employed by Noguchi and Sugiura (2001) for cyanobacteria, gave a faster Q_A^- oxidation reaction, by a factor of 7, but a much lower retention of oxygen evolution activity (38 ± 18%) after the measurement. DCBQ was added from a concentrated ethanol solution; the final ethanol concentration was ≤1%. A portion of the pellet was placed on a CaF_2 window, placed under a stream of nitrogen to concentrate the sample (~5 min), and a second CaF_2 window with a grease spacer was used to seal the sample. Before concentration, samples were either in a sucrose buffer, containing 0.4 M sucrose, 50 mM MES-NaOH, pH 6.0, and 15 mM NaCl, or in a glycerol buffer containing 5% glycerol, 50 mM MES-NaOH, pH 6.0, and 15 mM NaCl.

Actinic and preflashes were provided by a frequency-doubled, 532-nm output from a Surelight I or III Nd:YAG laser (Continuum, Santa Clara, CA). The pulse width was ~7 ns, and the pulse energy was 20–30 mJ cm^{-2}. FTIR data were collected on a Bruker (Billerica, MA) IFS-66v/S spectrometer, equipped with a MCT detector and a Harrick (Ossining, NY) temperature controller. The temperature was 4°C, and the spectral resolution was 8 cm^{-1}. A Happ-Genzel apodization function and four levels of zero filling were employed. A germanium filter blocked illumination of the sample by the internal HeNe laser of the FTIR spectrometer. FTIR data acquisition began 40 ms after the actinic flash, and the data were collected in 5-s data sets (34 mirror scans) for a minimum of 60 s. These data sets were ratioed to data recorded before the laser flash to give individual difference FTIR spectra. Data collected over 15 s were then averaged. Dark-minus-dark controls were constructed from 5-s data sets recorded before the actinic flash. All spectra were normalized to an amide II amplitude of 0.5 absorbance units. In Fig. 6 *A*, the spectra are an average of 10 difference spectra acquired from 10 samples. In Fig. 6 *B*, spectra are an average of 26 difference spectra acquired on 16 different samples.

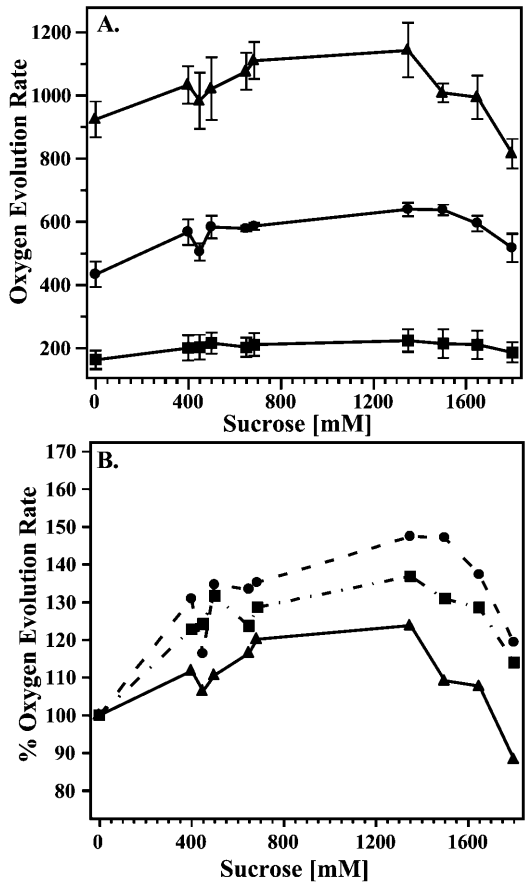

FIGURE 1 Sucrose effects on PSII oxygen evolution. Oxygen evolution assays were performed on intact (*triangles*), salt-washed (*circles*), and urea-washed (*squares*) PSII. *A* represents the oxygen evolution rate in μmol O_2 (mg chl h)$^{-1}$. In some cases, the error bars are smaller than the symbols used to present the data. *B* represents the data normalized as a percentage of the measured oxygen evolution rate at the first data point.

RESULTS

The steady-state oxygen evolving activity of PSII was monitored in varying concentrations of sucrose (Fig. 1) at 530 μM DCBQ. Oxygen evolution assays were performed on intact (Fig. 1, *triangles*), salt-washed (Fig. 1, *circles*), and urea-washed (Fig. 1, *squares*) PSII. In the range from 0 to 1.35 M sucrose, there was an increase in oxygen evolution rate from 920 ± 60 μmol O_2 (mg chl h)$^{-1}$ to 1,140 ± 90 μmol O_2 (mg chl h)$^{-1}$ (Fig. 1 *A*, *triangles*) in intact PSII. The sucrose-induced activity stimulation was followed by inhibition in the sucrose concentration range from 1.35 to 1.8 M (Fig. 1 *A*, *triangles*). Concentrations of 0.4 M sucrose are typically used for assay of oxygen evolution activity in PSII (Berthold et al., 1981). The data in Fig. 1 show that a significant stimulation of activity is observed at higher sucrose concentrations in intact PSII.

To evaluate the effect of the extrinsic proteins on these sucrose effects, PSII was salt-washed to remove the 18- and 24-kDa proteins (Ghanotakis et al., 1984a; Miyao and Murata, 1983a). PSII was also urea-washed to remove the manganese stabilizing protein, in addition to the 18- and 24-kDa proteins (Bricker, 1992; Miyao and Murata, 1983b). Both salt-washed and urea-washed PSII were found to require calcium and chloride for oxygen evolution activity, as expected (reviewed in Seidler, 1996). Also as expected, the overall rate of oxygen evolution declines by a factor of 2 to 3, even when calcium and chloride are present in the assay (Fig. 1 *A*). In salt-washed PSII, the steady-state oxygen rate was between 400 and 500 μmol O_2 (mg chl h)$^{-1}$ when no cosolvent was present (Figs. 1 and 2). This rate is in the range typically reported for salt-washed preparations (for example, see Ghanotakis et al., 1984b). In urea-washed PSII, the steady-state oxygen rate was ~150 μmol O_2 (mg chl h)$^{-1}$ when no cosolvent was present (Figs. 1 and 2). This rate is also in the range typically reported from urea-washed preparations (for example, see Bricker, 1992).

The observed dependence of oxygen evolution rate on sucrose concentration was not changed appreciably by removal of extrinsic subunits (Fig. 1 *A*, *squares* and *circles*). This is more obvious, when the rates are calculated as a percentage of the rate in the absence of sucrose (Fig. 1 *B*). Stimulation of activity was observed up to ~1.35 M sucrose for all three PSII preparations.

Steady-state oxygen activity was also monitored as a function of glycerol concentration in 530 μM DCBQ (Fig. 2). In intact PSII, addition of glycerol caused a stimulation of activity in the range from 0 to 30% (4.11 M) (Fig. 2 *A*,

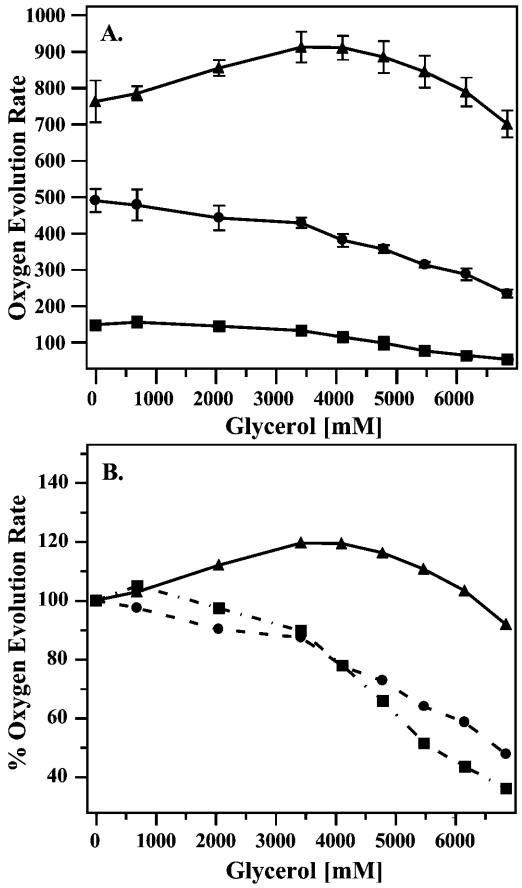

FIGURE 2 Glycerol effects on PSII oxygen evolution. Oxygen evolution assays were performed on intact (*triangles*), salt-washed (*circles*), and urea-washed (*squares*) PSII. *A* represents the oxygen evolution rate in μmol O_2 (mg chl h)$^{-1}$. In some cases, the error bars are smaller than the symbols used to present the data. *B* represents the data normalized as a percentage of the measured oxygen evolution rate at the first data point.

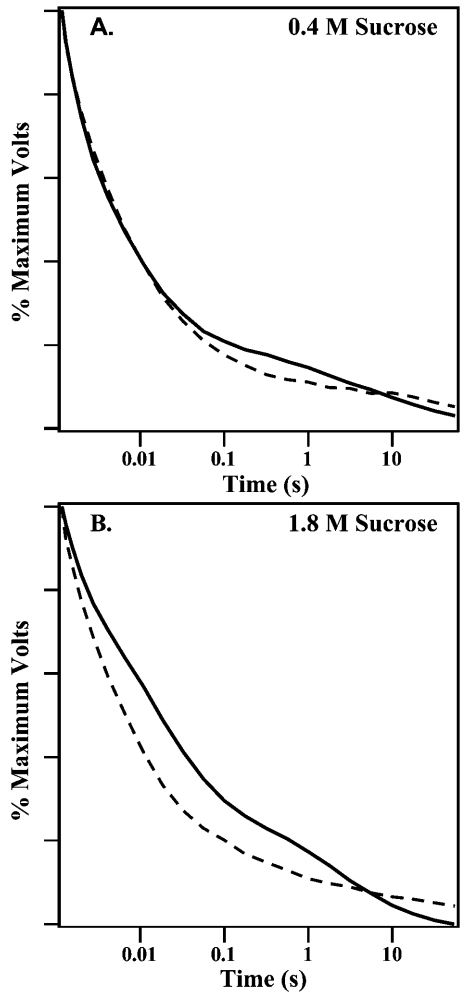

FIGURE 3 Sucrose effects on the rate of Q_A^- oxidation, as detected by the yield of chl fluorescence. *A* and *B* show the decay of chl fluorescence in intact PSII samples containing either 0.40 M (*A*) or 1.8 M (*B*) sucrose. In each panel, data shown in the dashed line was recorded in the presence of DCBQ (10 μM), and data shown in the solid line was recorded in the absence of DCBQ. Traces are normalized to the fluorescence yield at the first data point, which is 200 μs after the actinic flash.

triangles). At higher glycerol concentrations, an inhibition of activity was observed. In contrast to the effects observed in sucrose, the dependence of the steady-state rate on glycerol concentration is significantly altered when the extrinsic proteins are removed (Fig. 2 *A*, *circles* and *squares*). In salt-washed and urea-washed samples, the addition of glycerol inhibited activity at concentrations greater than ~0.5 M (Fig. 2, *A* and *B*). This difference is attributed to loss of the 18- and 24-kDa extrinsic proteins and to increased glycerol access to the PSII catalytic site (see Discussion section).

Figs. 1 and 2 demonstrate that sucrose and glycerol significantly alter the steady-state rate of oxygen production. As a first step in assessing whether these effects are mediated on the acceptor or donor side of PSII, chl fluorescence measurements were performed. In these experiments, the rate and halftime of Q_A^- reoxidation were monitored after an actinic flash by measuring the yield of chl fluorescence (Boerner et al., 1992). In these samples, Q_A^- decays by reduction of Q_B, which is expected to occur on the hundreds of microsecond to millisecond time scale (see de Wijn and van Gorkom, 2001, and references therein). Fig. 3 shows representative data, acquired on intact PSII in 0.4 M (Fig. 3 *A*) or 1.8 M (Fig. 3 *B*) sucrose. Data were collected in the absence (*solid line*) or presence (*dotted line*) of DCBQ. In 0.4 M sucrose (Fig. 3 *A*), DCBQ has no apparent effect on the fast phase of decay and only a minor effect on the slow phase. In 1.8 M sucrose (Fig. 3 *B*), the decay kinetics in the presence of DCBQ (*dashed line*) are similar to the kinetics observed in 0.4 M sucrose (Fig. 3 *A*, *dashed line*). However, in the absence of DCBQ and in 1.8 M sucrose (Fig. 3 *B*, *solid line*), an overall slowing of fluorescence decay is observed compared to the other three conditions. The reason for this effect at a very high sucrose concentration is unknown.

The derived halftimes for Q_A^- decay, as assessed by chl fluorescence yield, are plotted as a function of sucrose concentration in Fig. 4. In the presence of DCBQ (Fig. 4,

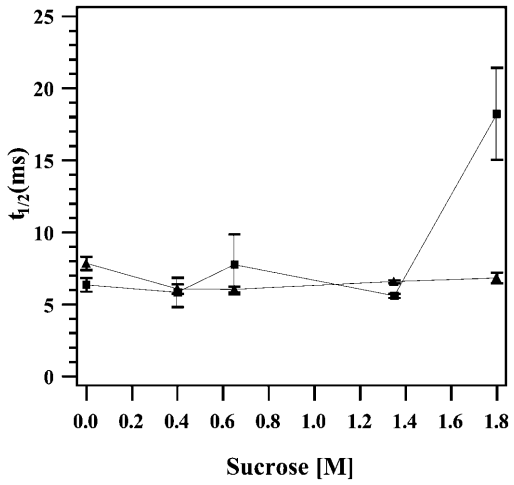

FIGURE 4 Sucrose effects on the overall halftimes for chl fluorescence decay in intact PSII samples. Data were obtained in the presence (*triangles*) or absence (*squares*) of DCBQ (10 μM).

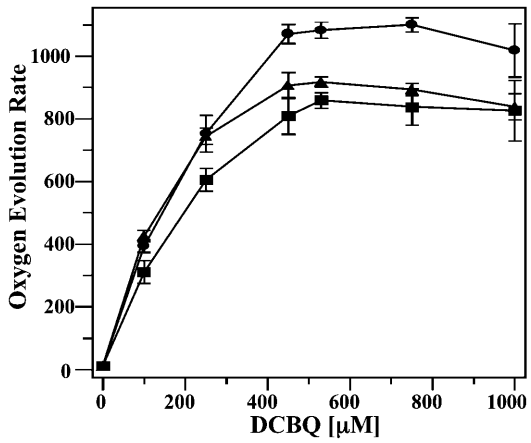

FIGURE 5 DCBQ effects on PSII oxygen evolution. Oxygen evolution assays were performed on intact PSII in 0.4 (*triangles*), 1.35 (*circles*), and 1.65 (*squares*) M sucrose.

triangles), the overall decay halftime is ~6 ms and is observed to be independent of sucrose concentration, given the standard deviation of the measurements. In the absence of DCBQ (Fig. 4, *squares*), the overall decay halftime is also ~6 ms and is independent of sucrose concentration, up to 1.8 M. At this concentration, the rate of chl fluorescence decay was observed to slow by a factor of ~3. These fluorescence data demonstrate that the overall rate of Q_A^- reoxidation is insensitive to sucrose concentration in the presence of DCBQ (compare to Fig. 1) and is insensitive to sucrose concentration in the absence of DCBQ up to a 1.8 M concentration.

Because sucrose and glycerol increase solvent viscosity, a slowing of a rate-limiting, second order rate of DCBQ reduction might explain the inhibitory phase of the concentration curves. Also, a decreased DCBQ K_M might explain a stimulation of oxygen evolution, if the K_M is on the same order of magnitude as the DCBQ concentration used in the oxygen evolution assays (530 μM). To test these possibilities, the dependence of oxygen evolution rate on DCBQ concentration was monitored. On a molar concentration basis, sucrose has more impact on viscosity, when compared to glycerol. For example, solutions containing either 1.5 M sucrose and 7 M glycerol have a relative viscosity of ~8, when compared to water at 20°C (CRC Handbook of Chemistry and Physics, 1972). Accordingly, DCBQ dependence was measured in three different concentrations of sucrose, where a significant viscosity effect would be expected (Fig. 5). The rate of oxygen evolution was observed to be dependent on DCBQ concentration at concentrations less than ~400 μM (Fig. 5). The rate was independent of DCBQ concentration in the range from 400 to 1,000 μM. Hyperbolic fits to the data showed that sucrose alters V_{max}, which is 1,360 μmol O_2 (mg chl h)$^{-1}$ in 1.35 M sucrose (*circles*) and 1,040 and 1,060 μmol O_2 (mg chl h)$^{-1}$ in 0.4 and 1.65 M sucrose, respectively (*triangles* and *squares*). A V_{max} of ~1,360 μmol O_2 (mg chl h)$^{-1}$ predicts a turnover time of ~10 ms. While sucrose-induced changes in V_{max} are observed, the V_{max} effect is not correlated with viscosity, because the V_{max} increased in 1.35 M sucrose, relative to 0.4 M sucrose.

Hyperbolic analysis of the data in Fig. 5 also gave the K_M for DCBQ, which has a value of 110–190 μM. This K_M is a factor of 5 lower than the DCBQ concentration used in the oxygen evolution assays (530 μM). At 530 μM, Fig. 5 shows that PSII is operating in the zero order regime; the rate does not depend on the DCBQ concentration. Also, the K_M was observed to be insensitive to sucrose concentration, given the standard deviation in the measurements (Fig. 5). For example, there is no evidence for a K_M decrease in 1.35 M sucrose, where the maximal oxygen evolution is observed. This experiment suggests that the observed stimulatory/inhibitory effects of sucrose are not due to a viscosity effect on a second order reaction or to a decrease in the K_M for exogenous acceptor. Taken together, the data in Figs. 3–5 suggest a donor-side, cosolvent effect.

To obtain more information concerning the effect of sucrose and glycerol on the PSII donor side, EPR spectroscopy was employed. Experiments were performed on salt-washed PSII; in these samples, the stimulatory and inhibitory effects of sucrose and glycerol, respectively, can both be investigated. In salt-washed PSII, the S_2 state, produced by illumination at 200 K, gives rise to the $g = 2.0$ multiline EPR signal (de Paula et al., 1986). In our experiments, the sucrose concentration was 0.4 and 1.35 M, and the glycerol concentration was 5% (0.68 M) and 30% (4.11 M). There was no significant change in the S_2 multiline lineshape under any of the four conditions; however, small changes in intensity were observed (data not shown). As reported previously (de Paula et al., 1986), no S_2 $g = 4.1$ signal was observed in salt-washed preparations, under any of the four conditions (data not shown).

Although the EPR experiments rule out a large structural change at the catalytic site, EPR spectroscopy is not sensitive to small structural changes, which might be significant, such as changes in hydrogen bonding, pK_A, and conformation. To investigate the possibility of small, but significant cosolvent-induced structural changes, reaction induced FTIR spectra, associated with $S_2Q_B^-$-minus-S_1Q_B, were acquired. Vibrational spectroscopy is exquisitely sensitive to changes in conformation and configuration in proteins. In these FTIR experiments, intact PSII was given a preflash, dark adapted for 1 h, and then given an actinic flash to photooxidize the S_1 state. Difference spectra were constructed from data collected (Fig. 6, *A* and *B*) after an actinic flash and before the actinic flash.

Fig. 6 *A* presents data acquired on a sample containing 5% (0.68 M) glycerol before concentration by a nitrogen flow. The final concentration of glycerol was estimated as 17% (2.3 M), based on the measured weight change (factor of 3.46). Fig. 6 *B* represents data acquired on a sample containing 0.40 M sucrose before concentration by a nitrogen flow. The final concentration of sucrose was estimated as 1.3 M, based on the measured weight change (factor of 3.14). These are sucrose and glycerol concentrations that stimulate oxygen evolution in intact PSII (Figs. 1 and 2). Note that EPR spectroscopy provided no evidence for orientation of PSII during the dehydration treatment.

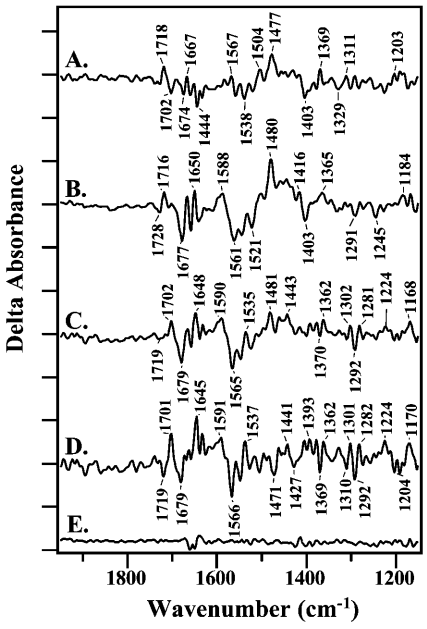

FIGURE 6 Light-minus-dark difference FTIR spectra, associated with $S_2Q_B^-$-minus-S_1Q_B, at 4°C in sucrose or glycerol. Difference spectra were constructed from data acquired immediately (0–15 s) after the actinic flash and from data acquired immediately before the flash. In *A*, the PSII samples were in a buffer containing 17% (2.3 M) glycerol. In *B*, the PSII samples were in a buffer containing 1.3 M sucrose. *C* shows the double difference spectrum constructed by a direct 1:1 subtraction of *A* from *B*, which were corrected for any small difference in protein concentration and pathlength. *D* shows the double difference spectrum obtained by an interactive subtraction of *A* from *B*, which was designed to minimize spectral contributions in the 1,800–1,200-cm^{-1} region. *E* shows a control spectrum constructed with data acquired before each actinic flash. On the *y* axis, the tick marks represent 2×10^{-4} absorbance units.

Significant differences are apparent when the difference FTIR spectra in Fig. 6, *A* and *B* are compared. The contributions from Q_B and Q_B^- will be equivalent under the two conditions, and the contributions from PSII quinone acceptors have been identified by isotopic labeling (Razeghifard et al., 1999) and time resolved spectroscopy (Zhang et al., 1998). Therefore, spectral differences in Fig. 6 arise from the donor side of PSII. Donor-side spectral assignments have been discussed (see for example Hillier and Babcock, 2001; Hutchison et al., 1999; Kim et al., 2000; Noguchi and Sugiura, 2001; Ono et al., 2001).

Fig. 6 *C* presents the double difference spectrum, constructed from a 1:1 subtraction of the 17% glycerol (Fig. 6 *A*) and 1.3 M sucrose (Fig. 6 *B*) data sets. The double difference spectrum in Fig. 6 *C* contain significant signals, compared to dark-minus-dark control spectra, constructed from data acquired before each actinic flash (Fig. 6 *E*). Significant signals were also observed when an interactive subtraction was used to minimize the overall intensity of contributions to the spectrum (Fig. 6 *D*). Note that the results of interactive (Fig. 6 *D*) and 1:1 (Fig. 6 *C*) subtraction are similar. These double difference spectra reveal the protein structural changes induced by cosolvent addition to intact PSII.

Addition of glycerol has the potential to change the spin state of the Mn cluster and convert the multiline signal to the $g = 4.1$ signal (Zimmermann and Rutherford, 1986). However, the multiline/$g = 4.1$ conversion does not occur in the presence of alcohols (Force et al., 1998), with the K_D for alcohol suppression estimated as ~60 mM. Because our samples contain 200 mM ethanol, a conversion from the multiline to the $g = 4.1$ S_2 state will not occur. Therefore, we attribute differential sucrose and glycerol FT-IR effects to changes in the S_2 multiline state.

In Fig. 6, *C* and *D*, alterations are observed in the 1,679–1,645-cm^{-1} and 1,566–1,535-cm^{-1} regions. These changes are candidates for amide I and II vibrational modes and may represent different conformations of the protein backbone (Bellamy, 1980). These spectral differences are attributed to a change in active site structure when glycerol and sucrose are compared. Free carboxylate groups and carboxylate ligands to Mn also make potential contributions in one or both of these spectral regions (Smith et al., 1997).

In Fig. 6 *D*, the spectral change observed at 1,719 (−)/1,701 (+) cm^{-1} is a derivative-shaped band, which may arise from the C=O stretch of one or more aspartic acid/glutamic acid side chains. The band is also observed in the 1:1 subtraction, Fig. 6 *C* and in the spectra before subtraction (compare Fig. 6, *A* and *B*). Observed spectral changes at 1,224 cm^{-1} may arise from the CO stretch of this functional group (Bellamy, 1980; Hutchison et al., 1999). If the aspartic or glutamic acid is near the active site, this amino acid side chain may be perturbed by a change in polarity, which can

generate a derivative-shaped band in the FTIR difference spectrum (see Steenhuis and Barry, 1997, and references therein). Such a change in polarity can occur because the hydration state of the active site is altered. Significant spectral differences are also observed in the 1,470–1,282 cm^{-1}. Bands in this region are attributable to the amide III band, free glutamic and aspartic acid residues, glutamic and aspartic acid ligands to Mn, and to other side chain vibrational modes, for example, those involving methyl rocking vibrations (Bellamy, 1980). Taken together, the observed spectral changes support the conclusion that a change in active site structure occurs, when glycerol- and sucrose-containing intact PSII are compared.

DISCUSSION

In this article, we show that addition of sucrose or glycerol stimulates the steady-state rate of oxygen evolution in intact PSII. This stimulation in activity is maintained up to 1.35 M sucrose or 30% glycerol and is attributable to an alteration in V_{max}. At higher concentrations, both cosolvents caused an inhibition of activity in intact PSII. In salt-washed and urea-washed samples, the effects of sucrose addition were similar to the functional dependence observed in intact PSII. However, glycerol was observed to be an inhibitor of oxygen evolution in the absence of the extrinsic subunits at low glycerol concentrations.

We attribute the effects of sucrose and glycerol to a donor side effect for the following reasons. First, the addition of sucrose had no effect on the overall rate of Q_A^- reoxidation, as assessed by chl fluorescence, in the presence of DCBQ. Second, the effects on the oxygen evolution rate are observed in a concentration regime in which the DCBQ binding site is saturated with acceptor, and electron transfer to DCBQ is likely to be rapid under these conditions. Third, when glycerol is used as a cosolvent, removal of the donor-side extrinsic subunits alters the concentration dependence. Fourth, difference FTIR spectroscopy detects structural differences, which are assignable to the donor side of PSII. This structural change consists of alterations in hydrogen bonding of the peptide backbone, possible structural changes at the active site, and a pK_A shift of one or more carboxylate groups.

Although this is the first report of these biphasic stimulatory/inhibitory effects on purified PSII, cosolvents and alcohols have been used to study PSII previously. For example, the effects of small alcohols (but not glycerol) were evaluated by a variety of magnetic resonance techniques (Force et al., 1998). Significantly for our interpretation, these previous results showed that both ethanol and methanol directly bind to the Mn cluster (Force et al., 1998). A thermoluminescence study has also been reported (Krieger et al., 1998). In that previous work, glycerol and other osmolytes were shown to alter the temperature at which luminescence, caused by S_2 recombination, was observed.

A previous study has examined the effects of ethylene glycol on PSII (Hillier et al., 1997). In the presence of this cosolvent, there was no stimulatory effect on oxygen evolution. This is not in contradiction to our results, because the effects of cosolvents can be quite cosolvent dependent (for example, see Sierks et al., 1997). At high concentrations, an inhibitory effect was attributed to loss of the extrinsic subunits (Hillier et al., 1997). A previous EPR study has suggested that glycerol can interact with the manganese cluster in intact PSII. The authors suggested that the addition of glycerol caused a change in the spin state of the Mn cluster, and this change in spin state was attributed to conformational differences at the catalytic site (Zimmermann and Rutherford, 1986).

Stimulatory effects of sucrose and glycerol on enzyme activity have been observed previously in other proteins. For example, addition of sucrose and glycerol stimulated calcium release from two isoforms of the ryanodine receptor. These effects were attributed to preferential hydration, which in turn led to increased ryanodine binding (Murayama et al., 1998). A biphasic pattern of stimulatory/inhibitory effects were also observed in a cosolvent study of the enzyme, glucoamylase (Sierks et al., 1997). Stimulatory effects were observed at low concentrations of polyethylene glycols; high concentrations caused inhibition. The authors concluded that, in the presence of stimulatory concentrations of cosolvent, the active site of glucoamylase is fixed in a conformation that allows more efficient release of the product.

To explain the biphasic effect of cosolvents on PSII activity, we consider the effects of cosolvent addition. Viscosity changes will occur. Increases in viscosity, due to the addition of glycerol or sucrose, have the potential to slow the rate of chemical reactions that are diffusion limited or involve large scale translational motion (Heimann et al., 2000). There is no electron or proton transfer reaction in PSII that is known to involve such a large-scale translational motion. A candidate reaction, for a diffusion-associated effect, might be the reduction of DCBQ on the PSII acceptor side. However, our oxygen evolution experiments were performed in a DCBQ concentration regime in which the DCBQ binding site is saturated. Further, our experiments showed that sucrose had no effect on the K_M for DCBQ. Therefore, it seems unlikely that an increase in viscosity alone can account for the decrease in PSII activity. It seems even less likely that an increase in viscosity would increase the steady-state oxygen evolution rate, as observed in our experiments.

As osmotic agents, sucrose and glycerol lower the activity of water and change the hydration state of proteins (Davis-Searles et al., 2001). Changes in hydration can potentially alter the rate of photosynthetic water oxidation and can account both for stimulatory and inhibitory effects (Sierks et al., 1997). According to one model, sucrose stabilizes the native structure of proteins through preferential osmolyte exclusion, which in turn causes preferential protein hydration (Timasheff and Arakawa, 1997). According to another model, the effects of glycerol are due to a solvophobic effect. This

effect causes glycerol to move away from nonpolar regions into the bulk solvent and again leads to preferential hydration of the protein (Timasheff and Arakawa, 1997).

To explain our results, we propose that preferential hydration leads to structural changes in the water oxidizing complex in intact PSII. Such changes in hydration could stimulate the activity of the water oxidizing enzyme in the regime where the protein is stably folded and could produce a more active catalytic structure. For example, the rate of product oxygen release might be accelerated due to a structural change. To explain the inhibitory concentration rate, we propose that further increases in hydration lead to local destabilization of critical structural elements. Alternatively, the inhibitory phase might be due to destabilization of the functional association of some PSII subunits. However, we do not favor the interpretation that these subunits are the 18-, 24-, and 33-kDa proteins, because sucrose was observed to have similar effects in intact, salt washed, and urea-washed PSII.

Our FTIR data support the preferential hydration interpretation, because, with this technique, we observed cosolvent-induced structural changes in intact PSII. The observed structural changes are consistent with protein structural changes at the active site and also with polarity-induced shifts of the pK_A of a carboxylic acid group (Bellamy, 1980; Dioumaev and Braiman, 1995). The direction of the shift represents an increase in the pK_A of the carboxylic acid group in glycerol, compared to sucrose (Dioumaev and Braiman, 1995). This change in pK_A is particularly interesting, considering that glycerol addition has been shown to change the proton release pattern of PSII (Haumann et al., 1997). Frequencies in the $\sim$1,700-cm^{-1} range and perturbed pK_A values have been reported for carboxylic acid residues in bacteriorhodopsin (Braiman et al., 1988). These carboxylic acid residues are involved in light-induced proton transfer in that protein.

In salt-washed and urea-washed PSII, glycerol has an additional effect on activity, because inhibition of oxygen evolution is observed even at relatively low glycerol concentrations. Removal of the extrinsic subunits is known to increase access to the Mn cluster (Ghanotakis et al., 1984b; Tamura and Cheniae, 1985), and small alcohols are known to bind to Mn (Force et al., 1998). Therefore, we propose that glycerol-induced inhibition in extrinsic subunit-depleted PSII at low glycerol concentrations is the result of glycerol binding to the Mn cluster.

A previous PSII FTIR study has studied the effect of glycerol addition to samples, which already contain 0.4 M sucrose (Onoda et al., 2000), and concluded that the addition of cosolvent did not alter the spectrum, except for minor changes in the amide I region. However, the mixture of cosolvents, employed in that previous study, decreased the probability of detecting spectral changes. Also, double difference spectra were not reported, so potential, interesting spectral changes may not have been identified in that work (Onoda et al., 2000).

Previously, variation in the FTIR spectrum associated with the S_1 to S_2 transition has been attributed to the presence of high concentrations of glycerol instead of sucrose in the preparation (Barry, 2000; Hutchison et al., 1999; Steenhuis and Barry, 1997). The work presented here supports the idea that the addition of cosolvents alters the FTIR spectrum and the structure of the active site.

This work was supported by National Science Foundation (NSF) grant MCB 0134968.

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