Different Manganese Binding Sites in Photosystem II Probed by Selective Chemical Modification of Histidyl and Carboxylic Acid Residues†

Ann Magnuson*,‡ and Lars-Erik Andréasson

Department of Biochemistry and Biophysics, Göteborg University and Chalmers University of Technology, Medicinaregatan 9C, S-413 90 Göteborg, Sweden

Received August 27, 1996; Revised Manuscript Received January 14, 1997⊗

ABSTRACT: The binding of Mn^{2+} to manganese-depleted photosystem II was investigated after chemical modification of histidyl and carboxylic acid residues in the presence or absence of the native manganese cluster. K_M values for Mn^{2+} were determined from steady-state electron transfer between Mn^{2+} and 2,6-dichlorophenolindophenol, the dissociation constant for Mn^{2+} was measured by observing the effect of added Mn^{2+} on the reduction of the primary donor $P680^+$ after a saturating flash, and single-turnover electron donation from Mn^{2+} was followed by monitoring the decay kinetics of the EPR signal from the flash-induced tyrosine Z^{ox} radical. K_M values for Mn^{2+} were found to be highly pH-dependent in both modified and unmodified photosystem II membranes. Treatment with histidine modifiers after removal of the manganese complex increased the K_M values between 2.5 and 10 times and increased the dissociation constant for Mn^{2+} 8-fold, compared to membranes that were modified in the presence of the manganese cluster. Modification of carboxylic acid residues after removal of the manganese cluster increased the K_M about 5-fold compared to membranes that were modified in the presence of the manganese cluster. The reduction rate of tyrosine Z^{ox} by Mn^{2+} was diminished after modification of either histidine or carboxylic acid residues. The apparent second-order rate constant decreased from 2.6×10^6 M^{-1} s^{-1} to 0.05×10^6 M^{-1} s^{-1} after histidine modification in the presence or absence of manganese, to 0.77×10^6 M^{-1} s^{-1} after carboxylic acid residue modification in the presence of manganese, and to 0.18×10^6 M^{-1} s^{-1} after carboxylic acid modification in the absence of manganese. Our results indicate the existence of two different manganese binding sites containing histidine, and at least two manganese sites with carboxylic acid residues, which are differently shielded against modifying agents by the native manganese cluster.

The transport of electrons from water to plastoquinone in photosystem II (PSII)[1] is carried out by the cofactors associated with the heterodimer of the core proteins D1 and D2. Water oxidation is assisted by four manganese ions in the water oxidizing complex (WOC), which is located close to the water−protein interface on the lumenal side of PSII. Three extrinsically bound protein subunits on the lumenal side stabilize the manganese cluster and promote water splitting activity. Light-induced oxidation of the primary electron donor chlorophylls, P680, leads to transfer of electrons to the components on the acceptor side, the primary acceptor pheophytin and the quinones Q_A and Q_B. Uniquely for PSII, in comparison to other photosynthetic reaction centers, are two redox active tyrosines, one in each of the D1 and D2 proteins. The oxidized P680 is re-reduced by tyrosine Z (TyrZ) in the D1 protein, which receives electrons extracted from water by the manganese complex. Four consecutive electron abstractions lead to one turnover of the WOC and the oxidation of two water molecules resulting in the release of molecular oxygen as a byproduct, a process in which the four manganese ions of the WOC serve as storage for accumulated positive charge through the four redox states of each turnover.

In recent years, much information has been collected about the chemical environment of the manganese cluster [see Debus (1992) for a review], but a detailed picture of the association between the manganese complex and the proteins of PSII is not yet available. EXAFS data imply that terminal manganese ligands, provided by the surrounding protein matrix, are oxygen and/or nitrogen atoms [see, e.g., Klein et al. (1993) for a recent review]. ENDOR and pulsed EPR studies have revealed nitrogen couplings to the manganese cluster in the S_2 state (De Rose et al., 1991; Tang et al., 1993), which have been shown to originate from a histidine at an as yet unidentified position (Tang et al., 1994). Hence, the efforts to identify the ligating amino acids have been focused on carboxylic acid and histidine residues, and site-directed mutagenesis on the D1 and D2 proteins as well as the chlorophyll binding proteins CP47 and CP43 has been employed to investigate possible manganese binding amino acid residues. The results from such mutagenesis studies

† This study was supported by grants from the Swedish Natural Science Council and the Sven and Lily Lawski Foundation.

* Author to whom correspondence should be addressed.

‡ Present address: Department of Biochemistry, Chemical Center, Lund University, Box 124, S-221 00 Lund, Sweden.

⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1997.

[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DEPC, diethyl pyrocarbonate; EDC, 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide; EDTA, ethylenediaminetetraacetate; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; Mes, 2-(*N*-morpholino)ethanesulfonic acid; P680, primary electron donor chlorophyll of PSII; PSII, photosystem II; TMAOH, tetramethylammonium hydroxide; Tris, tris(hydroxymethyl)aminomethane; TyrZ, redox active tyrosine of the D1 protein; WOC, water oxidizing complex of PSII; Q_A, Q_B, primary and secondary quinone acceptors.

S0006-2960(96)02176-9 CCC: $14.00

have implicated the involvement of His 190, His 332, and His 337 in the D1 protein (Diner et al., 1991; Kramer et al., 1994; Chu et al., 1995a,b.). In addition, two carboxylates in the D1 protein have been tentatively identified as probable manganese ligands, namely, Asp 170 (Diner & Nixon, 1992) and the C-terminal Ala 344 (Nixon et al., 1992). Recently, the possible involvement of several other residues has been discussed, e.g., Glu 65, Glu 189, Glu 333, and Asp 342 (Chu et al., 1995a,b). Nevertheless, it is not known exactly how many carboxylates or histidines are involved in the binding of manganese to PSII.

Chemical modification of amino acids has been used as an alternative method to site-directed mutagenesis in attempts to identify ligands to manganese. Especially, reagents specific for modifying histidines and carboxylic acid residues have been used for this purpose. In earlier studies (Tamura et al., 1989; Preston & Seibert 1991a,b) with this approach, the modification was conducted in the absence of the manganese cluster. These investigations suggested that histidyl residues are involved in photoactivation, the process in which the manganese cluster is assembled in a light-dependent reaction (Tamura et al., 1989). Carboxylates and histidines were furthermore found to be important for the electron donation by Mn^{2+} to PSII (Seibert et al., 1989; Preston & Seibert, 1991a,b).

In the absence of the manganese complex, extraneous Mn^{2+} is an efficient electron donor to PSII, and it is likely that at least some of the sites involved with electron donation are the natural sites for ligation of manganese. Electron donation by Mn^{2+} can be studied by steady-state electron transfer measurements, involving multiple turnovers, or in single turnover experiments. Single turnover assays include measuring $TyrZ^{ox}$ reduction by EPR spectroscopy (Hoganson et al., 1989) or following $P680^{+}$ reduction by optical or fluorescence spectroscopy (Conjeaud & Mathis, 1986; Hoganson et al., 1991). Multiple turnover has been investigated with dichlorophenolindophenol (DCIP) as an electron acceptor (Klimov et al., 1982).

In this study, we have examined the effects of chemical modification of carboxylates and histidines, performed either in the presence or in the absence of the manganese cluster, on steady-state and single turnover electron transfer. With this strategy, we have been able to discriminate between residues shielded by the manganese cluster against chemical modification, and more extrinsically located residues involved in binding of added manganese. Metal ions in proteins are to a large extent bound to charged amino acid residues, and, therefore, it can be expected that the binding affinity for manganese in PSII is pH dependent. Hsu et al. (1987) investigated Mn^{2+} as an inhibitor of electron donation by diphenyl carbazide (DPC) at two different pH values, and found that the K_M value for Mn^{2+} in this assay was higher at higher pH. With Mn^{2+} as the sole electron donor, however, little is known about its efficiency in correlation to pH. We have studied the pH dependency for binding of and electron donation by Mn^{2+}, in combination with chemical modification, to reveal the nature of the amino acids involved.

Our results indicate the involvement of both histidine and carboxylate residues susceptible to chemical modification, as well as indications for the presence of these amino acids at different manganese binding sites.

MATERIALS AND METHODS

Biochemical Preparations. PSII-enriched membranes were prepared from spinach as described by Franzén et al., (1985). The extrinsic regulatory proteins with molecular masses of 17, 24, and 33 kDa were removed by suspending the membranes in 1 M $CaCl_2$ for 30 min at 4 °C (Hsu et al., 1987; Tamura & Cheniae, 1987), followed by washing 3 times in 25 mM Mes−NaOH, pH 6.5, 10 mM NaCl, and 350 mM sucrose (buffer A), or 25 mM Mes−NaOH, pH 6.5, 20 mM NaCl, 20 mM $CaCl_2$, and 350 mM sucrose (buffer B) depending on further treatment as described below. The PSII membranes were either depleted of their native manganese prior to chemical modification or modified in the presence of the manganese cluster. In control samples, the native manganese was extracted after the modification was completed. Native manganese was removed after reduction with hydroquinone as described by Ghanotakis et al., (1984), slightly modified as follows: PSII membranes were suspended at 5 mg of Chl/mL in buffer A containing 2 mM hydroquinone and 10 mM EDTA, incubating for 75 min at 4 °C, and washing 3 times in buffer A. Alternatively, manganese was removed by incubating the membranes in 0.8 M Tris, pH 8.2, at 4 °C in room light. The residual manganese content in the depleted membranes was found to be 0.5−0.8 manganese per reaction center assuming a chlorophyll content of 250 Chl/PSII.

Chemical Modification. Chemical modification of histidyl residues was carried out in buffer B as described by Tamura et al., (1989) with the following modifications: The chlorophyll concentration was 0.5 mg/mL, corresponding to approximately 2 mM PSII centers, and the DEPC concentration was 7 mM. The reaction was stopped after 75 min, by the addition of 50 mM histidine in buffer A, and the membranes were washed 3 times in buffer A. Chemical modification of carboxylic acid residues with EDC was conducted mainly as described for the DEPC treatment. The EDC modification was conducted in darkness at 0.1 mg of Chl/mL, and the reaction was stopped after 60 min by the addition of 70 mM sodium acetate (Preston & Seibert, 1991a). The samples were stored at 77 K before they were used for the experiments described below. The manganese content was analyzed by denaturing the PSII membranes in 0.2 M H_2SO_4 and measuring the EPR signal from free Mn^{2+} (Franzén et al. 1985). The manganese content was 3.3−3.9 manganeses per PSII center immediately after removal of the extrinsic proteins, and 2.1−2.2 manganeses per PSII after 1 h of incubation at room temperature.

Steady-State Electron Transfer Measurements. PSII membranes were suspended at room temperature and 0.01 mg of Chl/mL in 20 mM Mes or Hepes buffer at pH values from 5.4 to 8.0, to which $MnCl_2$ was added from stock solutions. Mn^{2+} donation to PSII was monitored by DCIP photoreduction, measured with an Aminco-DW2 spectrophotometer in the dual-wavelength mode (Seibert et al., 1989; Preston & Seibert, 1991a). Saturating actinic light from a halogen lamp was lead into the sample compartment perpendicular to the measuring beam after passing through a heat filter and a cut-off filter (RG 630). The DCIP reduction rate was measured from initial slopes of the difference between absorbancies at 522 and 570 nm. Apparent K_M values for Mn^{2+} were determined from the *x*-axis intercept in Hanes plots ($[Mn^{2+}]$/reduction rate) vs $[Mn^{2+}]$).

Time-Resolved EPR Measurements. PSII membranes were suspended at 2 mg of Chl/mL in buffer A containing 2 mM $K_3Fe(CN)_6$ and 2 mM $K_4Fe(CN)_6$ at different concentrations of $MnCl_2$. The electron donation to $TyrZ^{ox}$ by Mn^{2+} (Hoganson et al., 1989) was measured in a flat cell at room temperature, with a Varian E9 EPR spectrometer equipped with a TM110 cavity, by monitoring the disappearance of signal II_{fast} after a 3 μs xenon flash with a flash interval of 4 s. During measurements, the magnetic field position was set on the low-field peak of signal II_{slow}, and a spectrometer time constant of 3 ms and a microwave power of 10 mW were used. The modulation frequency and amplitude were 100 kHz and 5 G, respectively. The kinetic traces were recorded with a Nicolet 490 digital oscilloscope. The reduction rate of $TyrZ^{ox}$ for each concentration of added Mn^{2+} was calculated by nonlinear least-squares fitting of an average of 200−400 traces, using a curve-fitting program based on the Levenberg−Marquardt algorithm. The signal amplitude was constant through several hundred flashes and did not change with the Mn^{2+} concentration.

Optical Measurements of $P680^+$ Reduction. PSII membranes were suspended at 0.1 mg of Chl/mL in a buffer containing 1 mM citrate, 30 mM Hepes−TMAOH, pH 7.4, 30% glycerol, 250 mM DCBQ (Hoganson et al., 1991), and different concentrations of Mn^{2+}. Flash-induced absorption transients from $P680^+$ were measured at 830 nm after excitation flashes from a frequency-doubled Nd/YAG laser, with a flash interval of 5 s. The instrumentation was essentially as described by Hoganson et al., (1991), but the output from the photodiode was recorded with a Nicolet 490 digital oscilloscope. At each Mn^{2+} concentration, an average of 16 traces was analyzed with the same method as the time-resolved EPR data.

RESULTS

Steady-State Electron Transfer Experiments. In PSII membranes, stripped of their native manganese complex, photoreduction of DCIP by Mn^{2+} was investigated after treatment of the membranes with a histidine modifier, DEPC, or a carboxylic acid modifier, EDC. In contrast to several earlier studies, where manganese binding was studied by observing the ability of added Mn^{2+} to inhibit electron donation from diphenyl carbazide (DPC) [e.g., Hsu et al., (1987) and Preston and Seibert (1991)], we have examined electron donation from Mn^{2+} directly. In particular, we have investigated the ability of the native manganese cluster to protect amino acids from modification, by comparing the effect of chemical modification in the presence of the manganese cluster and in its absence.

The electron donation from Mn^{2+} in chemically modified samples, from which manganese had been removed either prior to or after modification, was compared with identical measurements in manganese-depleted, but otherwise unmodified PSII membranes. Apparent K_M values for Mn^{2+} in manganese-depleted PSII were determined at different pH values from Hanes plots of the kinetics observed from photoreduction of DCIP by Mn^{2+} (Figure 1). The apparent K_M value for Mn^{2+} was found to be strongly dependent of pH (Figure 2), decreasing with increasing pH, which indicates an increasing affinity for Mn^{2+} at higher pH values. Below pH 7.0, we observed a biphasic manganese dependence of the turnover rate, with two apparent K_M values for

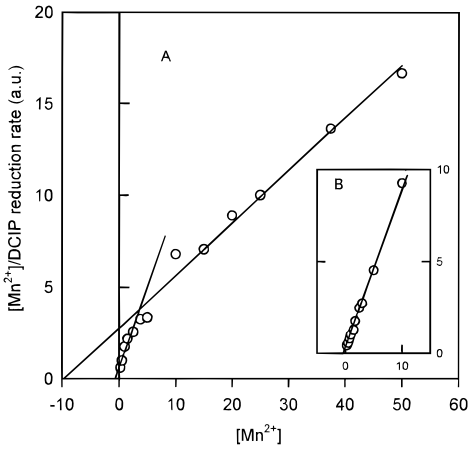

FIGURE 1: Examples of primary plots of kinetic data obtained from steady-state assays of light-induced DCIP reduction with Mn^{2+} (in micromolar) as electron donor. Measurements at pH 6.4 (A) and 8.0 (B, inserted) were carried out as described under Materials and Methods.

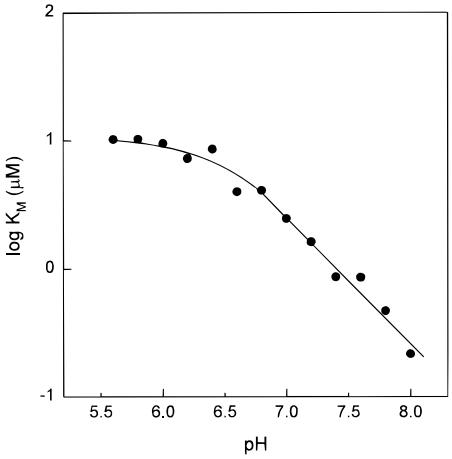

FIGURE 2: pH dependency of log K_M for Mn^{2+} in unmodified, manganese-depleted PSII membranes. The data are partly fitted to a straight line with a slope of −1.

Mn^{2+} (Figure 1). The lower K_M value, corresponding to a high-affinity site, was in unmodified membranes found to be 0.1−0.2 μM. This lower K_M value was not dependent on pH and contributed to less than 20% of the maximal rate at any pH below 7.0. In contrast, the higher K_M value, corresponding to a site with lower affinity, was found to vary strongly with pH. The K_M was 10 μM at pH 6.0 and changed to less than 1 μM at higher pH. The pH dependency, plotted as log K_M versus pH, fitted well to a straight line with a slope of −1 between pH 8.0 and 6.2, below which the pH dependency curve leveled off (Figure 2). The change of K_M, by a factor of 10 for each pH unit, is to be expected if the change represents the deprotonation of a single binding group.

Blubaugh and Cheniae (1990) observed a site for binding and electron donation by Mn^{2+}, with an even higher K_M of 200 μM which they ascribed to a site for unspecific electron donation where bound Mn^{2+} did not reduce TyrZ directly. For this reason, we have chosen not to study the electron transport at very high concentrations of Mn^{2+}, and in the following discussion we will deal with the pH-dependent binding site with K_M in the intermediate range. We have chosen to concentrate on the K_M rather then the k_{cat} value, since it should reflect events mainly on the donor side of PSII and since it is insensitive to variations in the extinction

coefficient of DCIP at different pH values, and uncertainties in the concentration of photosynthetic material.

Experiments were conducted with two types of chemically modified samples. One type consisted of PSII membranes chemically modified in the presence of the native manganese. In these samples, the extrinsic regulatory proteins were dislodged prior to the chemical modification. The manganese content of the PSII membranes immediately after removal of the extrinsic proteins was 3.2−3.9 manganese ions per 250 Chl, and 2.1−2.2 after 1 h of incubation at room temperature, which is to be expected when the stabilizing 33 kDa protein is absent [e.g., see Ono and Inoue (1984) and Kubawara et al., (1985)]. Thus, only two manganese ions per PSII center remained during the modification procedure, and some of the native manganese binding sites were exposed. After the modification, the native manganese was extracted, and the electron donation from extraneous Mn^{2+} was measured.

In the second type of sample, the PSII membranes were subjected to chemical modification after removal of both the natively bound manganese and the extrinsic regulatory proteins. Both types of chemically modified samples showed reduced turnover rates for steady-state electron transfer compared to unmodified membranes (data not shown). However, a comparison of the two different types of modified material should compensate for any global effects of chemical modification, e.g., effects involving the acceptor side, and shows only differences resulting from whether modification was conducted in the presence or absence of the manganese cluster.

Both types of DEPC-modified PSII membranes displayed apparent K_M values for Mn^{2+} which were dependent on pH and increased with decreasing pH. In this respect, they were both similar to the unmodified samples. The pH dependency was linear between pH 8.0 and 6.2 with a slope of −1, reaching a plateau below pH 6.2 with a corresponding value for the K_M of about 10 μM (Figure 3A). In fact, the K_M value for Mn^{2+} in the material modified in the presence of native manganese was indistinguishable from that in the chemically unmodified material at all pH values. In contrast, in the samples which had been DEPC-treated in the absence of native manganese, the apparent K_M value for Mn^{2+} was higher throughout the studied pH range, suggesting a generally lower affinity for Mn^{2+}. At pH values above 7.2, the pH dependency of the K_M clearly deviated from that observed in unmodified material and material modified in the presence of manganese, with the K_M leveling out at a constant value of about 1.5 μM. At pH 8.0, the K_M for Mn^{2+} was about 10 times higher than in the two other types of samples.

We also studied the effect of the carboxyl modifier EDC in a similar experiment. In samples modified with EDC in the presence of native manganese, the K_M value was the same as that in the unmodified samples between pH 8.0 and 6.2, but continued to increase below 6.2, suggesting the modification of a residue titrating below this pH value (Figure 3B). The K_M values for manganese in PSII samples modified in the absence of manganese also showed a linear pH dependence. However, the K_M values at pH 6.5 were about 5.5 and 3.9 times higher than in the chemically unmodified material and in the material modified in the presence of manganese, respectively. The pH dependency (Figure 3B) followed a straight line with slope −1 from pH 5.6 to 8.0.

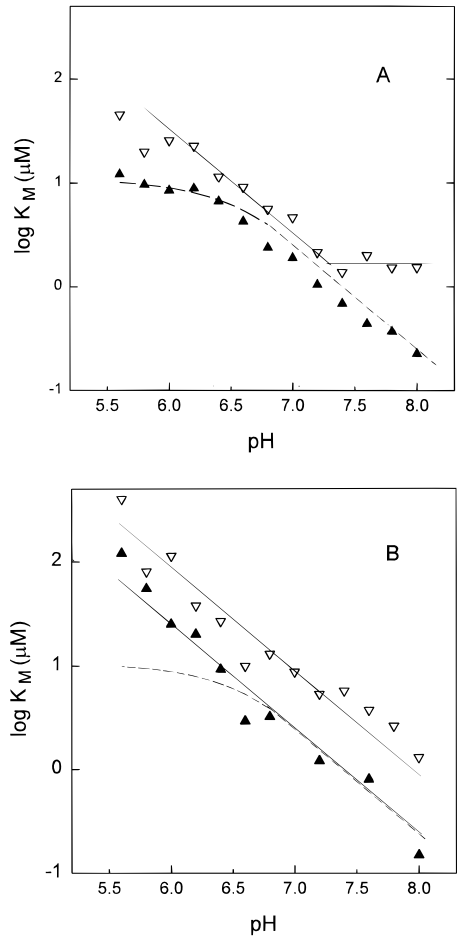

FIGURE 3: pH dependency of log K_M for Mn^{2+} in (**A)** DEPC-modified PSII membranes and (**B)** EDC-modified PSII membranes Samples modified in the presence of the native manganese cluster (▲) and membranes modified in the absence of manganese (▽). The dashed line shows the pH dependency in unmodified PSII membranes as in Figure 2.

Thus, in both types of EDC-modified PSII membranes, the pH dependency in the lower pH range was altered, and, in addition, the PSII samples modified in the absence of manganese had a notably lower affinity for Mn^{2+} over the whole pH range.

Time-Resolved EPR Measurements of Electron Donation to TyrZ. In manganese-depleted PSII membranes, the $TyrZ^{ox}$ reduction rate has previously been reported to increase by the addition of extraneous Mn^{2+}, due to electron donation by Mn^{2+} to $TyrZ^{ox}$ (Hoganson et al., 1989). At pH 6.5, this reduction rate follows pseudo-first-order kinetics, and can be fitted to a single exponential function. We made similar experiments both with unmodified, manganese-depleted PSII membranes and with PSII membranes modified with DEPC or EDC. With unmodified PSII membranes, our results were in agreement with Hoganson et al., (1989) (Table 1 and Figure 4A,B). In EDC-and DEPC-modified PSII material, the amplitude and intrinsic decay rate of the $TyrZ^{ox}$ radical were essentially the same as in unmodified material in the absence of extraneous Mn^{2+}, indicating that the redox properties of TyrZ were not altered by the modification. The ability of Mn^{2+} to reduce $TyrZ^{ox}$ was noticeably diminished in the DEPC-modified membranes modified either in the presence or in the absence of manganese compared to the unmodified material (Figures 4 and 5A). The corresponding second-order rate constant for reduction of $TyrZ^{ox}$ was 0.05

Table 1: Apparent Second-Order Rate Constants and K_M Values for Mn^{2+} in Manganese-Depleted PSII Membranes

	unmodified PSII	DEPC-modified in presence of Mn	DEPC-modified in absence of MN	EDC-modified in presence of Mn	EDC-modified in absence of Mn
app second-order rate constant (M^{-1} s^{-1})	2.6×10^6 ($\pm 0.05 \times 10^6$)	0.05×10^6 ($\pm 0.03 \times 10^6$)	0.05×10^6 ($\pm 0.02 \times 10^6$)	0.77×10^6 ($\pm 0.08 \times 10^6$)	0.18×10^6 ($\pm 0.07 \times 10^6$)
K_M value at pH 6.5 (μM)	5.3	4.2	10.3	7.1	27.6

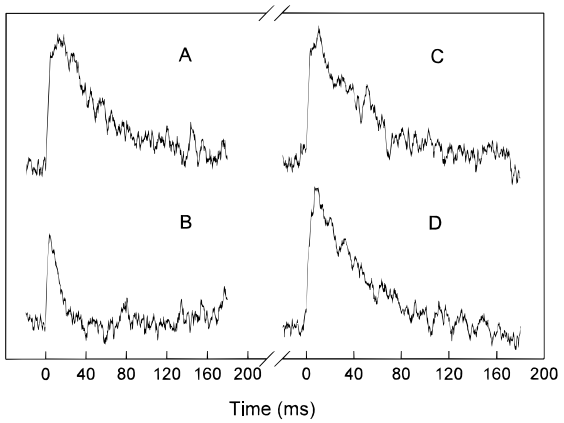

FIGURE 4: Decay in time of the amplitude of the $TyrZ^{ox}$ radical signal (signal II_{fast}) in the absence of exogenous electron donors, and in the presence of Mn^{2+}. (**A** and **B)** In unmodified PSII membranes and in (**C** and **D**) DEPC-modified material. (**A** and **C)** In the absence of exogenous Mn^{2+}; (**B** and **D)** after addition of 40 μM Mn^{2+}.

$\times 10^6$ M^{-1} s^{-1} in the DEPC-modified, and 2.6×10^6 M^{-1} s^{-1} in the unmodified material respectively (Figure 5A and Table 1). Thus, upon addition of 40 μM Mn^{2+}, the $TyrZ^{ox}$ reduction rate in unmodified PSII membranes increased 6 times while in the DEPC-modified membranes the rate was increased only by a factor 0.3 in the presence of manganese. When 1 mM Mn^{2+} was added to the DEPC-modified material, the reduction rate increased 3.5 times (data not shown). Interestingly, no significant difference in sensitivity to added manganese between the two types of DEPC-modified samples could be detected. Thus, the drastic decrease in the second-order rate constant for reduction by Mn^{2+}, after modification either in the presence or in the absence of manganese, indicates that the modification introduced considerable changes in the Mn^{2+} binding properties of the site, conceivably because residues involved in the binding of added manganese were exposed in both cases.

Modification of exposed carboxylic side chains by treatment of PSII membranes with EDC-treated PSII membranes also resulted in inhibition of the reduction of $TyrZ^{ox}$ by Mn^{2+} in both types of modified membranes, compared to the unmodified membranes (Figure 5B, Table 1). The effect was more pronounced in samples modified with EDC in the absence of manganese (Figure 5B, open triangles). The corresponding second-order rate constants decreased to 7.7×10^5 M^{-1} s^{-1} and 1.8×10^5 M^{-1} s^{-1}, respectively, as compared to 2.6×10^6 M^{-1} s^{-1} in the unmodified membranes. When 40 μM Mn^{2+} was added, the reduction rate for $TyrZ^{ox}$ increased by a factor 2 in the samples that were modified in the presence of the manganese cluster, and only by 0.5 in the material modified after the manganese was removed (Figure 5B). The difference between the two types of modified material suggests that additional modifiable carboxylic groups involved in binding of the added Mn^{2+} and in the electron donation from manganese to $TyrZ^{ox}$ were exposed by removal of the native manganese cluster.

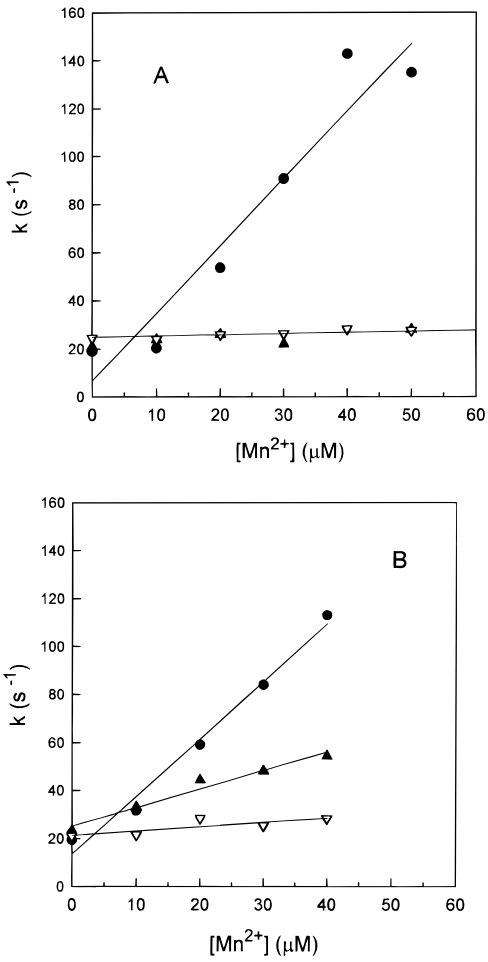

FIGURE 5: Apparent rate constants for $TyrZ^{ox}$ reduction, as a function of extraneous Mn^{2+} concentration, in **(A)** DEPC-modified PSII membranes and **(B)** EDC-modified membranes. Unmodified PSII membranes (●); samples modified in the presence of native manganese (▲); membranes modified in the absence of manganese (▽).

Optical Measurements of $P680^+$ Reduction. The time constant of the re-reduction of $P680^+$ by TyrZ in the absence of the manganese cluster is approximately 10 μs (Conjeaud & Mathis, 1981). This rate is affected by the presence of Mn^{2+}. Upon addition of Mn^{2+}, the 10 μs kinetic phase of the $P680^+$ reduction has been observed to gradually diminish in concert with the increase of a 40 μs phase (Hoganson et al., 1991). This result was explained by the electrostatic field imposed by the presence of bound Mn^{2+} ions, making TyrZ less prone to donate an electron to $P680^+$.

We conducted similar measurements (adding Mn^{2+} to manganese-depleted PSII) in PSII membranes modified with DEPC in the presence or absence of native manganese. In PSII membranes modified in the presence of manganese, the size of the 10 μs decay phase decreased significantly by addition of Mn^{2+}, or to less than 50% of the total signal amplitude after addition of 700 μM Mn^{2+}. However, in the membranes that had been modified after manganese extrac-

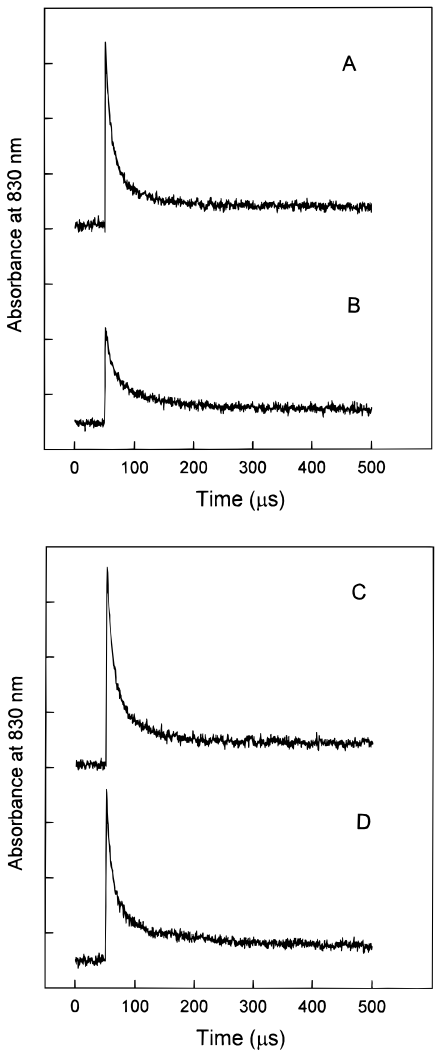

FIGURE 6: Traces of absorption transients at 830 nm in PSII membranes showing the kinetics for re-reduction of $P680^+$: (**A** and **B)** DEPC-modified in the presence of native manganese; and (**C** and **D)** DEPC-modified in the absence of manganese. (**A** and **C)** Without adding Mn^{2+}; (**B** and **D)** in the presence of 0.3 mM Mn^{2+}.

tion, after addition of 1 mM Mn^{2+} about 85% of the original signal amplitude of the 10 μs reduction phase remained (Figure 6). Figure 7 shows the relative amplitude of the 10 μs phase (fraction of the total signal amplitude) plotted as a function of the logarithm of Mn^{2+} concentration. Curve fitting revealed that the apparent dissociation constant for Mn^{2+} at the corresponding site at pH 7.4 was 90 μM in the samples modified in the presence of manganese and approximately 600 μM in the samples that were modified in the absence of native manganese cluster. Thus, chemical modification by DEPC in the absence of manganese lowered the affinity for extraneous Mn^{2+} in comparison to when modification was done before removal of bound manganese.

We observed two additional decay transients with time constants of 40 μs and 0.2−1 ms, respectively, but could not resolve any significant changes in the absolute amplitudes of these upon addition of Mn^{2+}. This contrasts to the observations by Hoganson et al., (1991) in unmodified material, where the decrease in the 10 μs phase was followed by a concomitant increase in the 40 μs kinetic phase. The observed decrease in the total signal amplitude was attributed to stacking of the PSII membranes in the presence of high

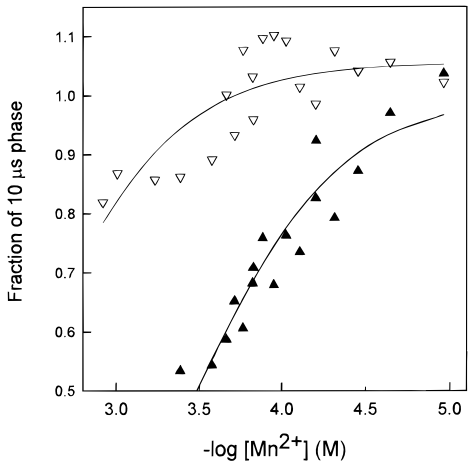

FIGURE 7: Fraction of 10 μs phase of the total kinetics for the reduction of $P680^+$, mirroring the ability of TyrZ to reduce $P680^+$, as a function of free [Mn^{2+}]. Samples modified with DEPC in the presence of the native manganese cluster (▲), and membranes modified in the absence of manganese (▽). The solid lines show sigmoidal dissociation curves that were fitted to the data. Apparent dissociation constants calculated from these were 90 μM in the samples modified in the presence of manganese, and 600 μM in samples modified in the absence of manganese.

concentrations of Mn^{2+}. In our experiments, we observed a decrease in the total signal amplitude on addition of Mn^{2+} in the samples modified in the presence of manganese, but not in the samples modified in the absence of manganese where a significant part of the amplitude remained. The discrepancies between our experiments and the observations by Hoganson et al., may suggest that the reduction mechanism for $P680^+$ reduction is more complex than previously thought. At any rate, it is clear from our results that the reduction kinetics of $P680^+$ in the two types of modified PSII membranes differ in sensitivity toward Mn^{2+} with regard to the 10 μs reduction kinetics. Obviously, the removal of the manganese cluster exposes histidine residues which are involved in the binding of the added Mn^{2+}, and which were not accessible to modification prior to manganese extraction.

DISCUSSION

Involvement of Histidines. Several reports suggest the involvment of histidine residues in the binding of the manganese cluster, but the exact number and location of these residues are not known. In our steady-state electron transfer measurements, the displayed pH dependence of the K_M for Mn^{2+} electron donation to PSII is that expected for the deprotonation of a single manganese binding residue in the pH range of 6.5 and above. In PSII membranes that have been DEPC-modified in the presence of native manganese, the pH-dependency curve of the K_M value for Mn^{2+} is very similar to that of the unmodified PSII membranes. In contrast, in the membranes that were modified in the absence of manganese, the K_M value is slightly higher in a large part of the pH range, and distinctly higher at pH values above 7.2 where the K_M becomes independent of pH. The lowered affinity for binding Mn^{2+}, as well as the loss of pH-dependency for the binding of Mn^{2+} above pH 7.2, is suggested to result from the modification of one or several Mn^{2+} binding histidyl residues which are exposed to DEPC when the manganese cluster is removed. Histidine binds metal ions in the deprotonated state, and a histidine residue with a pK_a value above the range of our measurements might

be responsible for the pH dependency of the K_M in unmodified membranes at higher pH values. Modification of this residue in the absence of the manganese cluster could account for the apparent loss of pH-dependency of the binding of Mn^{2+} by shifting the pK_a value from being above 8 to 7.2. Thus, from the differences in effects of DEPC on steady-state electron transfer from Mn^{2+}, we have evidence of a histidine that is exposed to modification only in the absence of the manganese cluster. This residue is likely to be a ligand to manganese in the intact water oxidizing complex.

We also investigated the effect of DEPC modification on the P680 reduction kinetics at pH 7.4, i.e., within the pH range where the difference in K_M is significant between the two types of modified material. These measurements showed that DEPC modification conducted in the presence of manganese results in an increase in the dissociation constant by almost a factor of 2 compared to that in unmodified membranes, and in the membranes modified in the absence of manganese the dissociation constant was almost 10 times higher than that reported for unmodified membranes (Hoganson et al., 1989). Thus, our measurements of P680 reduction corroborate the results from the steady-state measurements of DCIP reduction, that there are histidine residues that are protected by native manganese against DEPC modification. Moreover, the increase in dissociation constant after modification in the absence of manganese most likely accounts for the increase in the K_M value observed in the steady-state measurements.

The reduction of $TyrZ^{ox}$ by Mn^{2+} was severely impaired in the DEPC-modified PSII membranes compared to unmodified membranes, as reflected in the lowered apparent second-order rate constant. In apparent contrast to our other measurements, the effect from DEPC modification was almost identical in membranes that were modified in the presence and in the absence of native manganese, with nearly the same second-order rate constant for reduction by added Mn^{2+} in both types of DEPC-modified samples (Figure 5A, Table 1). This result suggests modification of a histidine residue which is not protected against modification by native manganese, and which contributes to the binding of the Mn^{2+} prior to electron donation to $TyrZ^{ox}$.

Comparing the observations from the $TyrZ^{ox}$ reduction assay, and the increases in K_M value and dissociation constant described above, it seems likely that there are two different manganese binding sites containing histidines. In both types of modification experiments, DEPC modifies a weak binding site for native manganese, which becomes exposed toward the modifier by removal of the manganese-stabilizing 33 kDa protein and the consequential loss of an average of two manganese per PSII center. Complete removal of all native manganese exposes a second site which binds native manganese more strongly. Modification of this site leads to the higher dissociation constant and K_M values we have observed.

When DEPC modification was done in the presence of native manganese, the K_M value was similar to that in unmodified material, while the dissociation constant and the rate constant for Mn^{2+} changed quite significantly. This manifests the common observation that other rate constants often influence the K_M value in addition to the dissociation constant. A decrease in the electron transfer rate on the donor side, due to a lower rate of electron donation to $TyrZ^{ox}$ from Mn^{2+}as described above, could compensate for an increase in the dissociation constant that otherwise would lead to an increase in the K_M value. The turnover rate for steady-state electron transfer was indeed observed to decrease after DEPC modification.

Nevertheless, there is a clear difference between the two types of modified material, and the K_M value should reflect differences in the substrate affinity between these. Thus, we suggest that the increase in K_M after modification in the absence of manganese, in comparison to the value observed after modification in its presence, is due to the increase in dissociation constant. Any effects of DEPC modification on the acceptor side should be identical in both types of modified samples and not affect the K_M value.

There is a considerable amount of site-directed mutagenesis work that may be used to aid in the identification of the two histidine-containing sites revealed in our chemical modification experiments. Mutations in D1 His 190 (Diner et al., 1991; Roffey et al., 1994a; Chu et al., 1995a) resulted in inactivation of water oxidation and inability to assemble the manganese cluster. His 190 was thus early on suggested to be a manganese ligand, and lately to be participating in redox reactions on the donor side by providing a hydrogen bond acceptor for TyrZ (Roffey et al., 1994a,b; Chu et al., 1995a). In *Chlamydomonas reinhardtii* His190Phe mutants lacking the manganese cluster, the K_M for Mn^{2+} at the high-affinity manganese site, for steady-state reduction of DCIP by Mn^{2+}, is apparently not any different from that in the wild type (Kullander et al., 1995). However, reduction of $TyrZ^{ox}$ by extraneously added Mn^{2+} could not be resolved in flash-induced single turnover experiments in these mutants, as opposed to in wild type (C. Kullander, F. Mahmedov, and S. Styring, personal communication). These observations show striking similarity to our results in DEPC-modified PSII membranes, and suggest that part of the effects from our modification with DEPC may involve D1 His 190. In light of these observations His 190 should not be excluded as a candidate for being a manganese ligand.

Other histidine residues on the D1 protein have been shown to be important for the activity of the water splitting complex [see, e.g. Diner et al., (1991), Nixon and Diner (1992a), and Chu et al., (1995b)], but at this point our results do not permit any detailed discussion about the involvment of these.

Involvement of Carboxylates. Spectroscopic and steady-state kinetic data as well as mutagenesis experiments have earlier suggested that carboxylates are involved in the ligation of the manganese cluster. In our measurements of steady-state electron transfer using EDC-modified PSII membranes modified in the presence of the native manganese cluster, the K_M for Mn^{2+} continued to increase with decreasing pH in contrast to that in unmodified membranes. Above pH 6.2, however, these samples displayed a pH dependency for the K_M for Mn^{2+} that was similar to that in the unmodified samples. The deviation from normal pH dependency at low pH values is an indication that we have modified Mn^{2+} binding carboxylates which are not shielded by the native manganese, or which are exposed to EDC due to manganese release during the modification.

EDC modification of PSII membranes depleted of native manganese resulted in a noticeably higher K_M for Mn^{2+} than in unmodified PSII membranes throughout the whole pH range. In addition, the pH dependency for the K_M was linear in the whole pH range, similar to what we observed in

membranes that had been modified in the presence of manganese. Since the K_M values above pH 6.2 are similar in the unmodified membranes and in the membranes modified in the presence of manganese, whereas they are distinctly higher in the samples modified in the absence of manganese, this is an indication that one or more additional sites involving carboxylate groups are made accessible to EDC on removal of all manganese.

EDC modification in the presence as well as in the absence of native manganese led to a decreased ability for Mn^{2+} to reduce $TyrZ^{ox}$. This effect, however, was much more pronounced in the membranes modified in the absence of manganese, resulting in a greatly reduced electron transfer rate from Mn^{2+} to $TyrZ^{ox}$. This supports our conclusion from the steady-state measurements, that there are at least two manganese binding carboxylate residues. Since EDC modification in the absence of manganese has the largest inhibitory effect on the electron donation by Mn^{2+}, it appears that the carboxylate with the "tightest" manganese binding, i.e., the one protected by native manganese against modification, is the one most important for reduction of $TyrZ^{ox}$.

Participation of carboxylates in the binding of Mn^{2+} involved in the electron transfer from Mn^{2+} to TyrZ supports results from site-directed mutant studies. Especially mutations in D1-Asp170 in *Synechocystis* PCC6803 (Nixon & Diner, 1992) and *Chlamydomonas reinhardtii* (Whitelegge et al., 1995), as well as D1-Glu189 (Chu et al., 1995a,b), have resulted in effects similar to ours. In D1-Asp170 mutants, the oxygen evolving activity was inhibited, and the K_M for reduction of $TyrZ^{ox}$ by exogenous Mn^{2+} increased as the pK_a increased for the amino acid at that substituted Asp. Mutations of Glu 189 resulted in a slower electron transfer from manganese to $TyrZ^{ox}$, which suggests that Glu189 can contribute to the redox activity of TyrZ (Chu et al., 1995a,b). We observed similar effects of EDC modification in PSII membranes in the absence of manganese, indicating possible involvement of Asp 170 and Glu 189 in our measurements.

Concluding, our results from chemical modification experiments of PSII complement site-directed mutagenesis studies in the identification of manganese ligating amino acids in the water oxidizing complex. By using a procedure which allows us to specifically modify those residues that are normally protected by natively bound manganese, we have obtained results that strongly indicate the involvement of both imidazoles and carboxylates as ligands. In addition, the different effects of chemical modification on parameters such as the electron transfer rate for Mn^{2+} and the affinity for added Mn^{2+} suggest the possibility to discriminate between different binding amino acids of a particular class. A natural extension of this work would be to identify the amino acid residues that are susceptible to chemical modification, by applying these techniques to mutants of suspected amino acid residues in the D1 and D2 proteins. Furthermore, although the D1 and D2 proteins are generally considered to be the subunits responsible for binding manganese, the possible involvement of other proteins has not been investigated fully.

ACKNOWLEDGMENT

We thank Dr. Örjan Hansson for most valuable assistance with the time-resolved optical measurements. Prof. Stenbjörn Styring is acknowledged for helpful and inspiring discussions.

REFERENCES

Blubaugh, D. J., & Cheniae, G. M. (1990) *Biochemistry 29*, 5109−5118.

Chu, H.-A., Nguyen, A. P., & Debus, R. J. (1995a) *Biochemistry 34*, 5839−5858.

Chu, H.-A., Nguyen, A. P., & Debus, R. J. (1995b) *Biochemistry 34*, 5859−5882.

Conjeaud, H., & Mathis, P. (1980) *Biochim. Biophys. Acta 590*, 353−359.

Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.

DeRose, V. J., Yachandra, V. K., McDermott, A. E., Britt, R. D., Sauer, K., & Klein, M. P. (1991) *Biochemistry 30*, 1335−1341.

Diner, B. A., Nixon, P. J., & Farchaus, J. W. (1991) *Curr. Opin. Struct. Biol. 1*, 546−554.

Franzén, L. G., Hansson, Ö., & Andréasson, L.-E. (1985) *Biochim. Biophys. Acta 808*, 171−179.

Ghanotakis, D. F., Topper, J. N., & Yocum, C. F. (1984) *Biochim. Biophys. Acta 767*, 524−531.

Ghirardi, M. L., & Seibert, M. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. II, pp 357−360, Kluwer Academic Publishers, Dortrecht, The Netherlands.

Hoganson, C. W., Ghanotakis, D. F., Babcock, G. T., & Yyocum, C. (1989) *Photosynth. Res. 22*, 285−293.

Hoganson, C. W., Casey, P. A., & Hansson, Ö. (1991) *Biochim. Biophys. Acta 1057*, 399−406.

Hsu, B.-D., Lee, J.-Y., & Pan, R.-L. (1987) *Biochim. Biophys. Acta 890*, 89−96.

Klein, M. P., Sauer, K., Yachandra, V. K. (1993) *Photosynth. Res. 38*, 265−277.

Klimov, V. V., Allakhverdiev, I. S., Shuvalov, V. A., & Krasnovsky, A. A. (1982) *FEBS Lett. 148*, 307−312.

Kramer, D. M., Roffey, R. A., Govindjee, & Sayre, R. T. (1994) *Biochim. Biophys. Acta 1185*, 228−237.

Kullander, C., Fredriksson, P. O., Saure, R. T., Minagva, J., Crofts, A. R., & Styring, S. (1995) in *Photosynthesis: From Light to Biosphere* (Mathis, P., Ed.) Vol. II, pp 321−324.

Kuwabara, T., Miyao, M., Murata, T., & Murata, N. (1985) *Biochim. Biophys. Acta 806*, 283−289.

Miller, A. F., & Brudvig, W. (1990) *Biochemistry 29*, 1385−1392.

Nixon, P. J., & Diner, B. A. (1992a) *J. Cell Biochem. 16D*, 118.

Nixon, P. J., & Diner, B. A. (1992a) *Biochemistry* 31, 942−948.

Nixon, P. J., Trost, J. T., & Diner, B. A. (1992) *Biochemistry 31*, 10859−10871.

Ono, T., & Inoue, Y. (1984) *FEBS Lett. 168*, 281−286.

Preston, C., & Seibert, M. (1991a) *Biochemistry 30*, 9615−9624.

Preston, C., & Seibert, M. (1991b) *Biochemistry 30*, 9625−9633.

Roffey, R. A., van Wijk, K. J., Sayre, R. T., & Styring, S. (1994) *J. Biol. Chem. 269*, 5115−5121.

Roffey, R. A., Kramer, D. M., Govindjee, & Sayre, R. T. (1994) *Biochim. Biophys. Acta 1185*, 257−270.

Seibert, M., Tamura, N., & Inoue, Y. (1989) *Biochim. Biophys. Acta 974*, 185−191.

Styring, S., Miyao, M., & Rutherford, A. W. (1986) *Biochim. Biophys. Acta 890*, 32−38.

Tamura, N., & Cheinae, G. (1987) *Biochim. Biophys. Acta 890*, 179−194.

Tamura, N., Ikeuchi, M., & Inoue, Y. (1989) *Biochim. Biophys. Acta 973*, 281−289.

Tang, X.-S., Sivaraja, M., & Dismukes, G. C. (1993) *J. Am. Chem. Soc. 115*, 2382−2389.

Tang, X.-S., Diner, B. A., Larsen, B. S., Gilchrist, M. L., Lorigan, G. A., & Britt, R. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 704−708.

BI962176C