Azide as a Competitor of Chloride in Oxygen Evolution by Photosystem II†

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Received December 30, 1998; Revised Manuscript Received March 18, 1999

ABSTRACT: Oxygen evolution by higher plants requires chloride, which binds to a site associated with the oxygen-evolving complex of photosystem II (PSII). In this study, the inhibitory effect of the anion azide was characterized using steady state measurements of oxygen evolution activity in PSII-enriched thylakoid membranes. N_3^- (7.8 mM) inhibited O_2 evolution activity by 50% when a standard buffer containing chloride was used. By considering Cl^- as the substrate in O_2 evolution assays, we found azide to be primarily competitive with Cl^- with an inhibitor dissociation constant K_i of about 0.6 mM. An uncompetitive component with a K_i' of 11 mM was also found. Removal of the 17 and 23 kDa polypeptides resulted in a decrease in each inhibition constant. A pH dependence study of O_2 evolution activity showed that the pH maximum became narrower and shifted to a higher pH in the presence of azide. Analysis of the data indicated that an acidic residue defined the low side of the pH maximum with an apparent pK_a of 6.7 in the presence of azide compared with 5.5 for the control. A basic residue was also affected, exhibiting an apparent pK_a of 7.1 compared with a value of 7.6 for the control. This result can be explained by a simple model in which azide binding to the chloride site moves negative charge of the anion away from the basic residue and toward the acidic residue relative to chloride. As a competitor of chloride, azide may provide an interesting probe of the oxygen-evolving complex in future studies.

Oxygen evolution by higher plants and algae takes place within photosystem II (PSII)[1] via a mechanism involving successive oxidations of the oxygen-evolving complex (OEC), which contains a cluster of manganese ions (*1*, *2*). According to the Kok (S state) model, four oxidation steps of the OEC from state S_0 to S_4 are driven by the energy derived by photon absorption at the reaction center of PSII. When the highest oxidation state (S_4) is reached, molecular oxygen is evolved and the OEC returns to the lowest oxidation state (S_0). Electrons that are removed from water in the oxidation cycle of the OEC are shuttled to plastoquinones Q_A and Q_B, which are in association with a nonheme Fe(II).

Chloride has long been known to be a necessary cofactor for oxygen evolution (*3*, *4*). PSII-enriched thylakoid membranes exhibit the best rates of activity in the presence of millimolar quantities of chloride. Other anions do not serve as well and can activate PSII with decreasing effectiveness in the following order: $Cl^- > Br^- \gg NO_3^- > I^-$. The requirement for chloride is apparently mediated by the extrinsic proteins with masses of 17 and 23 kDa. Recent studies have shown that chloride binds in several OEC S states, including S_1-S_3, but is more readily exchanged in the higher oxidation states. The affinity for Cl^- is 1 order of magnitude lower in the S_2 than in the S_1 state (*5*). Also, advancement from S_2 to S_3 and from S_3 to S_4 was found to require Cl^-, while advancement from S_0 to S_1 and from S_1 to S_2 did not (*6*).

Azide (N_3^-) has also been found to be in inhibitor of oxygen evolution in previous studies (*7*, *8*). When PSII membranes are illuminated in the presence of an electron acceptor, the inhibition takes a form that is not reversible, as shown by removing azide after treatment (*8*). After Tris treatment of PSII-enriched membranes to remove manganese, an azidyl radical was spin trapped under conditions of catalytic turnover, suggesting that azide was oxidized by the tyrosine Y_Z radical. The authors hypothesized that the irreversible inhibition of PSII was due to subsequent damage to PSII caused by the azidyl radical. The lack of a spin-trapped azidyl adduct using manganese-containing PSII-enriched membranes was thought to be a result of a poor exchange with the medium when the OEC was intact.

In the study presented here, the effects of azide on the initial rates of oxygen evolution have been investigated, thereby focusing on the initial binding site of azide. We have found that azide is primarily a competitive inhibitor with respect to chloride activation. In addition, we have characterized the effects of azide on the pH dependence of O_2 evolution activity.

EXPERIMENTAL PROCEDURES

PSII-enriched thylakoid membranes were prepared from market spinach by extraction with Triton X-100 as originally described by Berthold, Babcock, and Yocum (*9*) and later modified (*10*). Preparations were suspended in a final buffer

† This work was supported by grants from the National Science Foundation (MCB-9600925) and Research Corp. (CC4049).

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[1] Abbreviations: OEC, oxygen-evolving complex; PPBQ, phenyl-*p*-benzoquinone; PSII, photosystem II; S_0-S_4, oxidation states of the OEC according to the Kok model; SDS, sodium dodecyl sulfate.

10.1021/bi983075c CCC: $18.00
Published on Web 04/22/1999

of 20 mM Mes (pH 6.3), 0.4 M sucrose, 15 mM NaCl, and 5 mM $MgCl_2$, and frozen in liquid N_2 for storage at a concentration of 5−10 mg of chl mL^{-1}.

Removal of the polypeptides with masses of 17 and 23 kDa was carried out by incubation of the PSII-enriched membranes in a buffer containing 20 mM Mes (pH 6.3), 0.4 M sucrose, 15 mM $CaCl_2$, and 1.5 M NaCl for 45 min on ice under room lights. The NaCl-treated membranes were then washed with the same buffer but without the 1.5 M NaCl. Removal of the polypeptides was confirmed by SDS−polyacrylamide gel electrophoresis (*11*), using gels containing 13% acrylamide and 3 M urea. O_2 evolution activity after treatment was decreased to about 89% of the control in the presence of sufficient Ca^{2+} and Cl^-.

O_2 evolution rates were measured at 25 °C with a Clark type O_2 electrode (Yellow Springs Instruments, model 5331) using phenyl-*p*-benzoquinone (PPBQ) (Eastman Kodak Chemicals) as the electron acceptor. For some experiments, the PPBQ had been purified by recrystallization from ethanol. The O_2 concentration was calibrated using sodium dithionite in water to set zero and distilled water equilibrated with air to set 250 μM. During assays, samples were illuminated by a Dolan-Jenner model 180 Fiber-lite high-intensity illuminator with dual gooseneck fiber optics cable and an EJV 21 V 150 W lamp using the maximum intensity setting. This provided a total intensity of >1800 μmol of photons m^{-2} s^{-1} (395 W m^{-2}), measured using an Oriel Goldilux GRP-1 light meter, which was saturating for typical O_2 evolution measurements. O_2 evolution rates of control samples were typically 300−400 μmol of O_2 (mg of chl)$^{-1}$ h^{-1} in the final suspension buffer using the unpurified PPBQ and 400−500 μmol of O_2 (mg of chl)$^{-1}$ h^{-1} using the purified PPBQ. For each O_2 evolution activity study described below, data were fitted by nonlinear least-squares analysis using the program SigmaPlot, version 4.0 (SPSS Inc.). Errors were based on standard deviations of multiple measurements (three or more per data point).

For the study of the concentration dependence of azide inhibition, O_2 evolution assays were performed in the final PSII suspension buffer. The PSII-enriched membrane sample was added to the buffer after the addition of PPBQ and NaN_3 when present. Data were fitted to a model assuming one type of N_3^- binding site and that PSII centers with N_3^- bound were inactive.

For the study of azide inhibition when chloride was treated as a substrate, the assay buffer contained 20 mM Mes (pH 6.3), 0.4 M sucrose, and 6 or 10 mM $CaSO_4$ for intact or NaCl-treated PSII-enriched membranes, respectively, and the indicated concentrations of NaCl and NaN_3. The PSII-enriched membrane sample was added to the buffer containing the given concentration of NaN_3 and preincubated for 2 min at 25 °C before the addition of PPBQ. Ca^{2+} was added to the assay medium to prevent the possible inhibitory effects of Na^+, which may be a concern for PSII-enriched membranes without the 17 and 23 kDa polypeptides. For membranes without these polypeptides, Na^+ was found in an earlier study to inhibit competitively at the Ca^{2+} binding site with a K_i of 5 mM, while Ca^{2+} was found to have a dissociation constant of about 30 μM (*12*, *13*). Thus, the addition of 10 mM Ca^{2+} would be sufficient to block essentially all Na^+ binding at this site.

Data from the experiments involving inhibition of chloride activation by azide were analyzed according to the methods of Cornish-Bowden and Dixon (*14*, *15*) to determine dissociation constants for competitive and uncompetitive binding of an inhibitor. This was carried out assuming the general model for enzyme kinetics, described by the reaction velocity, v:

$$v = \frac{V_{max}S}{K_m\left(1 + \frac{I}{K_i}\right) + S\left(1 + \frac{I}{K_i'}\right)} \quad (1)$$

where V_{max} is the maximum reaction velocity, K_m is the Michaelis constant, S is the substrate concentration, I is the inhibitor concentration, and K_i and K_i' are competitive and uncompetitive inhibitor dissociation constants, respectively. In a Dixon plot ($1/v$ vs I for various S values), the values of I and $1/v$ at which the lines intersect, I_{com} and $(1/v)_{com}$, are given by

$$I_{com} = -K_i \quad (2a)$$

$$\frac{1}{v_{com}} = \frac{1}{V_{max}}\left(1 - \frac{K_i}{K_i'}\right) \quad (2b)$$

Similarly, in a Cornish-Bowden plot (S/v vs I for various S values), the values of I and S/v at which the lines intersect, I_{com} and $(S/v)_{com}$, are given by

$$I_{com} = -K_i' \quad (3a)$$

$$\frac{S}{v_{com}} = \frac{K_m}{V_{max}}\left(1 - \frac{K_i'}{K_i}\right) \quad (3b)$$

These two plotting methods simplify the determination of the inhibitor dissociation constants and also offer a way of estimating values of V_{max} and K_m for the substrate. In the experiments presented here, those assays involving PSII-enriched membranes without the 17 and 23 kDa polypeptides were carried out using PPBQ that was more pure than that used for assays involving intact PSII-enriched membranes. It is important to note that although the purity of PPBQ affects the overall rates of O_2 evolution activity and hence the slopes of the lines of the Dixon and Cornish-Bowden plots, the point of intersection of the data is not affected.

For the study of the pH dependence of azide inhibition, buffers for O_2 evolution assays were prepared with the same composition as the final suspension buffer except that the buffering reagent was varied as follows: itaconic acid, pH 5.2−5.8; Mes, pH 5.8−6.4; Pipes, pH 6.4−7.1; Mops, pH 6.9−7.7; Hepes, pH 7.2−7.8; and tricine, pH 7.9−8.4. Itaconic acid was compared with Mes at pH 5.8 to check for possible inhibitory effects, which were not evident. For each set of assays, measurements were taken using the final suspension buffer with Mes (pH 6.3) as a reference to define 100% activity. Data were fitted to a model involving two protonatable residues, in which the activity A depends on the $[H^+]$ as given by the equation

$$A = A_{max}\left(1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}\right) \quad (4)$$

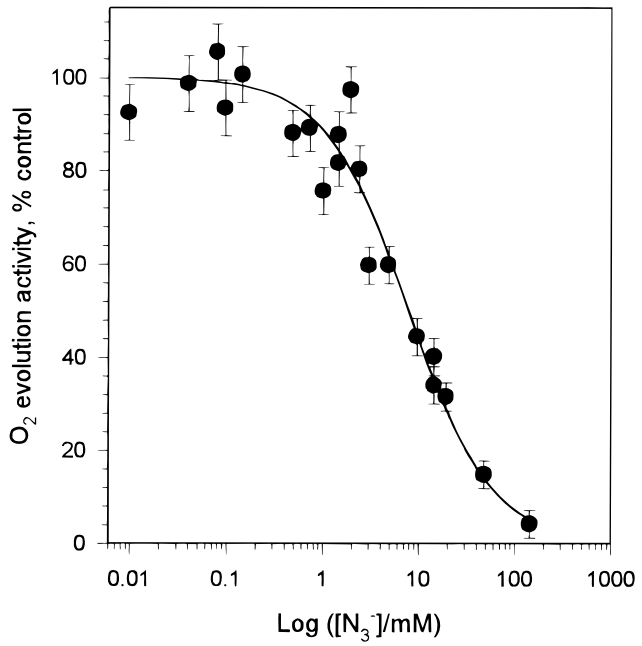

FIGURE 1: Effect of azide on the O_2 evolution activity of PSII-enriched membranes. Assays were carried out as described in Experimental Procedures in the final PSII suspension buffer, which contained 25 mM chloride. The curve shows a fit to the data that assumes inhibition resulted from binding to a single type of binding site.

where K_{a1} is the acid dissociation constant for the group that must be deprotonated for activity (acidic residue), K_{a2} is the acid dissociation constant for the group that must be protonated for activity (basic residue), and A_{max} is the maximum possible activity obtained when both groups are in their optimal states.

RESULTS

Inhibition by Azide. Addition of NaN_3 to PSII-enriched thylakoid membranes assayed in the final suspension buffer resulted in suppression of O_2 evolution activity. Analysis showed that the activity fit a simple logarithmic dependence on azide concentration (Figure 1). The concentration at half-maximal activity, $[N_3^-]_{50\%}$, was 7.8 mM in this buffer medium.

These data can be adequately modeled by assuming that azide bound to a single type of site, inactivating PSII centers to which it was bound. Given this model, the value for $[N_3^-]_{50\%}$ would be equal to the apparent dissociation constant $K_{d,app}$ for N_3^-. However, the number of azide binding sites under these conditions remains undetermined because of the very low concentration of PSII centers, which was estimated to be about 0.15 μM on the basis of a ratio of 200 chlorophyll molecules per PSII center.

To check for reversibility of azide inhibition under noncatalytic conditions, PSII-enriched membranes were incubated with various millimolar concentrations of NaN_3 on ice under low light. N_3^- was then removed by washing with the final suspension buffer. This resulted in a recovery of O_2 evolution activity to 95% or more of the control activity. In addition, treatment with NaN_3 was found to have no effect on the appearance of PSII-enriched membrane samples as determined by SDS−polyacrylamide gel electrophoresis.

Table 1: Effect of NaCl on the Inhibition of O_2 Evolution Activity Due to Azide[a]

sample treatment	O_2 evolution activity [μmol of O_2 (mg of chl)$^{-1}$ h^{-1}]	% activity
2 mM NaCl	345 ± 20	100 ± 6
2 mM NaCl and 5 mM NaN_3	172 ± 7	50 ± 4
7 mM NaCl	366 ± 22	100 ± 6
7 mM NaCl and 5 mM NaN_3	203 ± 8	56 ± 4
50 mM NaCl	391 ± 23	100 ± 6
50 mM NaCl and 5 mM NaN_3	220 ± 9	56 ± 4

[a] Assays were carried out as described in Experimental Procedures in a buffer containing 20 mM Mes (pH 6.3), 0.4 M sucrose, 6 mM $CaSO_4$, and the indicated concentrations of NaCl and NaN_3. One hundred percent activity is defined separately for each Cl^- concentration as that observed in the absence of added NaN_3.

Table 2: Effect of HCO_3^- on the Inhibition of O_2 Evolution Activity Due to Azide[a]

sample treatment	O_2 evolution activity [μmol of O_2 (mg of chl)$^{-1}$ h^{-1}]	% azide-free activity
no additions	292 ± 15	100 ± 5
7.5 mM NaN_3	210 ± 11	72 ± 5
10 mM HCO_3^-	268 ± 13	100 ± 5
10 mM HCO_3^- and 7.5 mM NaN_3	176 ± 7	66 ± 4
50 mM HCO_3^-	173 ± 7	100 ± 4
50 mM HCO_3^- and 7.5 mM NaN_3	92 ± 3	31 ± 3

[a] Assays were carried out as described in Experimental Procedures in a buffer containing 20 mM Hepes (pH 7.5), 0.4 M sucrose, 25 mM NaCl, and the indicated concentrations of $NaHCO_3$ and NaN_3. One hundred percent activity is defined separately for each HCO_3^- concentration as that observed in the absence of added NaN_3.

Competition of Azide with Chloride. The concentration of NaCl in the assay medium was found to affect the degree of inhibition by azide. When the chloride concentration was increased, the degree of inhibition by azide decreased (Table 1). Similar experiments performed by adding bicarbonate to the assay buffer did not show a relief of inhibition by azide (Table 2). Rather, bicarbonate seemed to enhance the inhibitory effect of azide. Thus, azide inhibition was relieved by chloride but not by bicarbonate.

The inhibition of O_2 evolution activity due to azide was analyzed considering chloride as a substrate by varying the concentrations of both azide and chloride in the assay buffer. Data were analyzed by the method of Dixon (*14*, *15*), plotting $1/v$ versus $[NaN_3]$, where v is the initial O_2 evolution rate. Simultaneous fits of the data gave a competitive inhibition constant K_i of 0.6 mM (Figure 2A) with a standard error of 0.5 mM; therefore, this inhibition constant should be taken as an estimate. Analysis of the same data by the method of Cornish-Bowden (*14*), plotting $[Cl^-]/v$ versus $[NaN_3]$, gave an uncompetitive inhibition constant K_i' of 11 mM (Figure 2B) with a standard error of 1 mM. These findings indicate that azide acted primarily as a competitive inhibitor toward chloride, preventing the binding of chloride at the OEC. These data furthermore provided estimates of the kinetic constants for the uninhibited reaction, including a K_m of 0.7 mM for chloride activation.

A similar set of assays was carried out using PSII-enriched membranes from which the 17 and 23 kDa polypeptides had been removed by incubation with 1.5 M NaCl. Removal of these polypeptides, which are thought to control access of

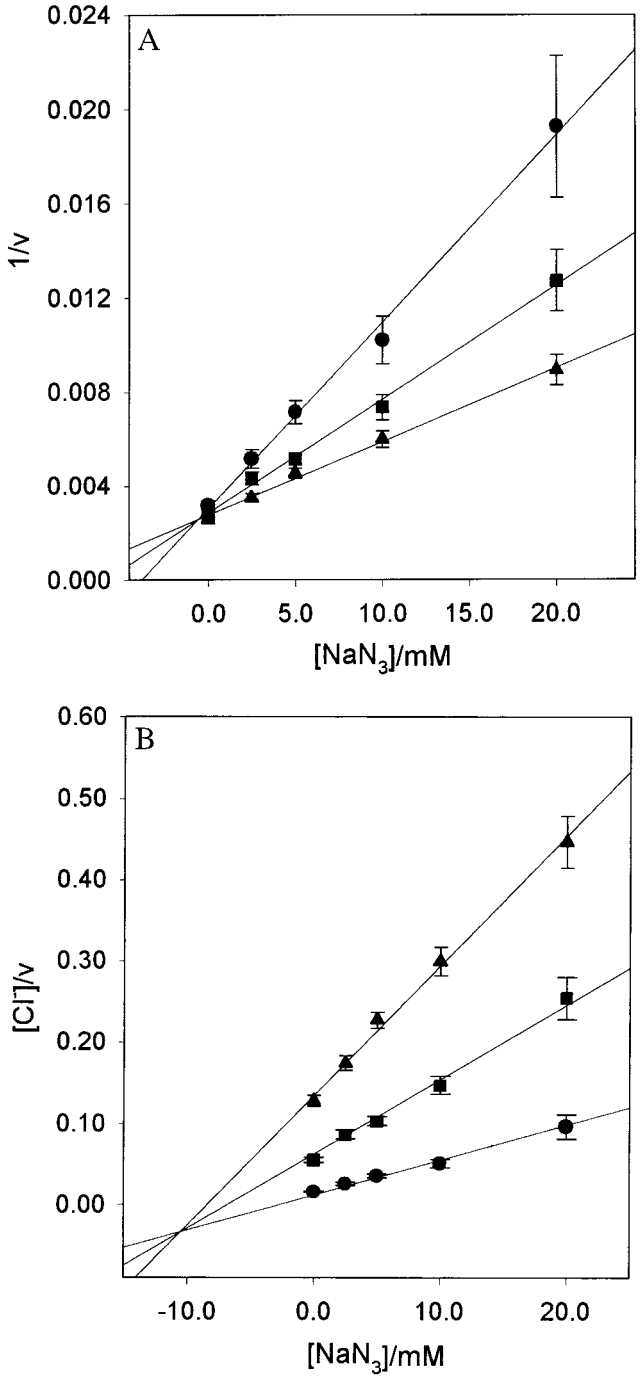

FIGURE 2: Dixon (A) and Cornish-Bowden (B) plots of azide inhibition of the O_2 evolution activity of PSII-enriched membranes, considering Cl^- as the substrate. The Cl^- concentrations were (●) 5, (■) 20, and (▲) 50 mM. Assays and data analysis were carried out as described in Experimental Procedures in the presence of the indicated concentrations of NaCl and NaN_3. The lines represent simultaneous linear fits to the data that required a common intersection point for all lines on each graph. For the Dixon plot, the intersection point gives the competitive inhibition constant $-K_i$ on the [NaN_3] axis; for the Cornish-Bowden plot, the intersection point gives the uncompetitive inhibition constant $-K_i'$ on the [NaN_3] axis.

Cl^- and Ca^{2+} to the OEC (*1*, *2*, *5*), is expected to have an effect on the anion binding constants at the OEC. In these experiments, Ca^{2+} was present at a concentration of 10 mM to prevent possible inhibitory effects of Na^+ at the Ca^{2+} binding site. The results of the Dixon analysis (Figure 3A) for NaCl-treated PSII-enriched membranes gave a competitive inhibition constant K_i of 0.26 mM for N_3^-, with a standard error of 0.12 mM. Results of the Cornish-Bowden analysis (Figure 3B) gave an uncompetitive constant K_i' of 2.3 mM, with a standard error of 0.2 mM.

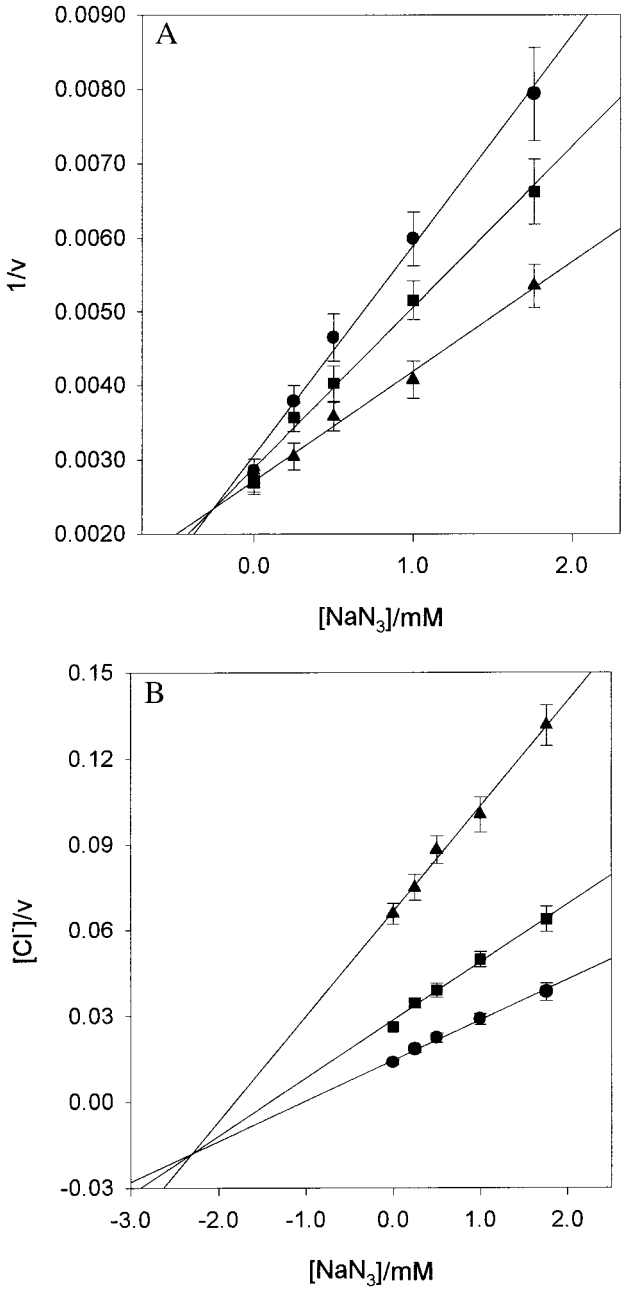

FIGURE 3: Dixon (A) and Cornish-Bowden (B) plots of azide inhibition of the O_2 evolution activity of PSII-enriched membranes without the 17 and 23 kDa polypeptides, considering Cl^- as the substrate. The Cl^- concentrations were (●) 5, (■) 10, and (▲) 25 mM. Assays and data analysis were carried out as described in Experimental Procedures. The lines represent simultaneous linear fits to the data, as described in the legend of Figure 2.

pH Dependence of Azide Inhibition. A study of the pH dependence of the inhibitory effect of azide was undertaken to help reveal information about the residues involved in N_3^- binding. The O_2 evolution activity was assayed over a pH range of 5.2−8.4 for both control PSII-enriched membranes and membranes in the presence of 7.7 mM NaN_3 (Figure 4). The degree of inhibition due to azide was found to vary as the pH varied, leading to changes in both the position and maximum of the pH dependence curve. The inhibition

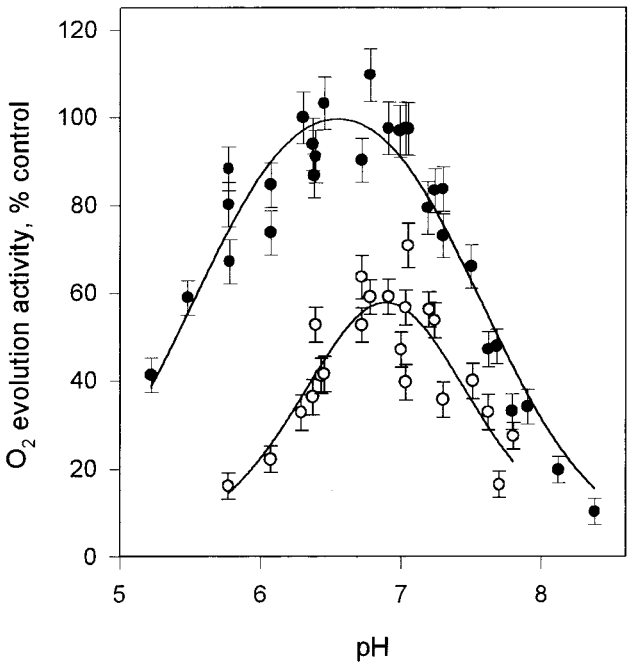

FIGURE 4: pH dependence of the azide inhibition of the O_2 evolution activity of PSII-enriched membranes: (●) control and (○) 7.7 mM NaN_3. Assays were carried out as described in Experimental Procedures in a buffer containing 25 mM Cl^-. One hundred percent activity was defined for each experiment as that measured at pH 6.3 in the standard final suspension buffer. Curves represent fits to the data assuming two protonatable functional groups with apparent pK_as of 5.5 and 7.6 for the control and pK_as of 6.7 and 7.1 in the presence of azide.

was more pronounced at lower pH. For example, the activity in the presence of 7.7 mM N_3^- was only 23% of the control at pH 6.1 compared with 47% of the control at pH 7.0.

A nonlinear least-squares fit to the pH dependence curve of control O_2 evolution activity assuming two protonatable residues gave a pK_{a1}(con) of 5.5 for the acidic residue and a pK_{a2}(con) of 7.6 for the basic residue. A fit to the data for the N_3^- -inhibited sample gave a pK_{a1}(N_3^-) of 6.7 and a pK_{a2}(N_3^-) of 7.1. Thus, the effect of azide was to replace both pK_as defining the pH maximum with more neutral values, thereby narrowing the pH range of activity. The theoretical maximum activities found for the fitted curves were as follows: A_{max}(con) = 119 ± 6% for the control and A_{max}(N_3^-) = 125 ± 41% for the azide-containing sample. (The higher error in the latter case is probably related to the fact that the optimal pH range was narrower with a lower observed maximum.) The theoretical maximum represents the activity with both residues in their optimal states and would only actually be observed if the two pK_a values were widely separated.

DISCUSSION

In the work of Kawamoto et al. (*8*), azide was shown to cause irreversible inhibition of O_2 evolution activity if the system was allowed to undergo catalytic turnover in the presence of azide. These investigators presented evidence that azide is oxidized to the azidyl form and then reacts with one or more unidentified residues within PSII. They also concluded that the site of inhibition caused by the photogenerated azidyl radical lies between Y_Z and Q_A. The study presented here is complementary to the work of Kawamoto and co-workers in that it describes the initial binding site for azide within photosystem II. We have focused on the initial rate of O_2 evolution using PSII-enriched membranes that have not previously undergone catalytic turnover. The work presented here therefore does not address the events that follow the initial binding of azide.

The inhibitory effect of azide on the O_2 evolution activity of PSII-enriched thylakoid membranes was studied to help classify it among the many anion and Lewis base inhibitors of this system. Inhibition by azide was notable at millimolar concentrations of NaN_3, with a half-inhibition constant of 7.8 mM at pH 6.3 in the presence of 25 mM Cl^-. The effects of azide were reversible, as long as the system was not allowed to undergo catalytic turnover, and no loss or alteration of polypeptides was evident during SDS−polyacryamide gel electrophoresis. Thus, the possibility that azide acted by extracting manganese or removing PSII polypeptides can be ruled out.

When analyzed as an inhibitor of chloride's participation in oxygen evolution activity, azide was found to be mainly competitive with chloride, indicating that it either binds at the chloride site or otherwise prevents binding of chloride. To confirm whether competition with chloride was generally responsible for inhibition by azide, it is important to compare the dissociation constants that are involved. A rough comparison can be made assuming that the inhibition by azide is due completely to competition with chloride, with a competitive dissociation constant for azide K_i of 0.6 mM. In this case, the K_d of Cl^- binding would have to have been around 2 mM for 50% of the activity to remain in the presence of 7.8 mM N_3^- and 25 mM Cl^-.

The literature supports a dissociation constant for Cl^- in the millimolar range under the conditions of catalytic turnover. In a recent study, the Cl^- concentration for half-maximal O_2 evolution activity (which is equivalent to the Michaelis constant K_m) was found to be 6.5 mM (*5*). This was interpreted as an overall dissociation constant K_d of 6.5 mM for Cl^- based on a simple binding equilibrium. In the same study, the binding of chloride was found to depend on the S state of the OEC, with the S_2 and S_3 states exhibiting much higher dissociation constants than the lower states. A similar trend was found in a study using NMR line width measurements of ^{35}Cl (*16*); this study also reported a half-maximal concentration of about 5 mM Cl^- for activation of O_2 evolution by PSII from mangrove. An earlier study using thylakoid membranes found that the half-saturation concentration of chloride was about 0.9 mM (*17*). Finally, in this study, K_m for activation by Cl^- was estimated to be about 0.7 mM. Although these constants vary somewhat, they support a K_d for Cl^- of ~2 mM and are therefore consistent with the interpretation that competition with chloride is the primary mode of inhibition by azide.

When spinach was grown on a medium enriched in ^{36}Cl, PSII was found to have one high-affinity binding site for chloride that is in slow exchange with the medium (*18*). The dissociation constant K_d was found to be about 20 μM, and 15−20 h of dialysis was required to remove bound chloride. It is noteworthy that this carefully measured K_d would be much too low to be the type of Cl^- site described here with a K_d in the millimolar range. However, this constant probably reflects the binding of chloride in the S_1 state and therefore

does not contradict an overall K_d in the millimolar range under conditions of catalytic turnover.

Some information regarding the location of the uncompetitive azide binding site can be gained from the experiments using PSII-enriched membranes from which the 17 and 23 kDa subunits of the OEC were removed by NaCl treatment. In these experiments, the competitive inhibition constant K_i was found to be 0.26 mM. This probably represents a decrease to about half of the value of K_i found for intact PSIIs, although the standard error in the latter value was somewhat large. The uncompetitive inhibition constant was also found to decrease substantially, with a K_i' of 2.3 mM. Reductions of this nature would be expected if removal of the 17 and/or 23 kDa polypeptides removed a barrier to the binding sites. These results therefore indicate that the uncompetitive as well as the competitive binding site for azide is at or near the OEC.

The latter result indicates that the uncompetitive binding site was probably not associated with binding of azide to the Fe(II)$-Q_A$ site on the acceptor side of PSII. Binding of azide at the Fe(II)$-Q_A$ site in place of bicarbonate had been suggested in an earlier study involving the effects of several anions on the slowing of oxidation of Q_A^- observed in chlorophyll *a* fluorescence decay measurements in *Synechocystis* 6803 (*19*). However, the effect of azide was not clear since 5 mM bicarbonate only partially reversed the slowing due to 100 mM azide, compared to complete reversal of slowing due to formate and nitrite. In the study presented here, relief of the azide inhibition of oxygen evolution by the addition of bicarbonate was tested to check for evidence that azide bound to the Fe(II)$-Q_A$ site. Such relief was not found; rather, the presence of bicarbonate seemed to enhance the inhibitory effect of azide (perhaps through inhibitory effects of its own on the donor side of PSII).

As an inhibitor of oxygen evolution that is primarily competitive with chloride, azide is in the same category of inhibitors as fluoride and amines other than ammonia (*20*, *21*). Of these, F^- and Tris base exhibited relatively large competitive constants K_i of ~4 and 12−14 mM, respectively. 2-Amino-2-ethylpropanediol exhibited a K_i value of 0.8 mM, and methylamine and *tert*-butylamine each exhibited K_i values of 0.02−0.03 mM. Each of these also exhibited a component of uncompetitive inhibition, but the uncompetitive constant was at least 10-fold greater than the competitive constant. Ammonia itself exhibited two modes of inhibition, with a K_i of 0.2−0.4 mM and a K_i' of 0.5−0.6 mM, and is believed to bind at both the chloride and water binding sites of the OEC. The results of the studies of Sandusky and Yocum showed that competition with chloride binding is not restricted by the size of the Lewis base. This suggests either a very wide pocket for the chloride site or that amines block access of Cl^- to its binding site without directly binding themselves.

Azide was found to have a notable effect on the pH dependence of the O_2 evolution activity of PSII. The two proton dissociation constants found for the control curve [pK_{a1}(con) = 5.5 and pK_{a2}(con) = 7.6] were replaced with more neutral values in the presence of 7.7 mM NaN_3, resulting in a narrowing of the pH optimum. The shift in the apparent pK_a was found to be most pronounced for the acidic side, with an apparent $pK_{a1}(N_3^-)$ of 6.7, although a significant effect was also observed for the basic side, with an apparent $pK_{a2}(N_3^-)$ of 7.1. The apparent pK_as found in the presence of azide may represent a mixture of PSII centers with and without azide bound to the Cl^- competitive site. Also, the altered pK_a values may represent a change in the pK_a of residues other than those that define the control pH dependence curve. The similarity in the theoretical maximum activities (A_{max}) found for both pH dependence curves suggests that the decrease in activity is accounted for by a shift in activating pK_as, and does not require the postulation of a change in mechanism that might reduce the maximum activity A_{max}. It is also notable that the similar values of A_{max} in the presence and absence of azide indicate that a decrease in activity due to the loss of manganese was not significant.

The possibility that the inhibitory form of azide was actually hydroazoic acid, HN_3, was considered since the shift of the pH maximum in O_2 evolution activity might be explained if an inhibitory form was removed as a result of deprotonation. Calculation shows that the inhibitor was overwhelmingly in the deprotonated azide form at the pHs used for this study (e.g., the ratio $[N_3^-]/[HN_3] = 50$ at pH 6.3). In addition, the pK_a for hydroazoic acid is 4.6, which is well below the apparent $pK_{a1}(N_3^-)$. Thus, the possibility that HN_3 was the inhibitory form was ruled out.

Previous studies have reported the influence of both acidic and basic residues in chloride binding. In a study of the pH dependence of the binding of $^{36}Cl^-$ to chloride-depleted PSII-enriched membranes, the extent of binding was found to drop at pHs of >7.5, consistent with the involvement of a residue with a pK_a of about 7.5 (*22*). If Cl^- binding does involve a residue that becomes deprotonated with a pK_a of ~7.5 (which would be equivalent to displacement of Cl^- by OH^-), we may have observed a shift in the apparent pK_a of the same residue to 7.1. In another earlier study, Homann found using chloride-depleted PSII-enriched membranes that chloride activation was controlled by groups with pK_as of <5 (*23*). These residues may be those observed here to shift to an apparent pK_a of 6.7 in the presence of azide. The participation of two types of protonatable residues (acidic and basic) in chloride binding does not imply that there are two chloride or azide binding sites.

The interpretation of the shifts in the apparent pK_as to more neutral values in the presence of azide may be complicated by the effects of increasing pH, since OH^- is thought to be a possible competitor of Cl^-. The displacement of Cl^- by OH^- may result in a decrease in the apparent affinity for Cl^-. This effect would have been minimized by the fairly high Cl^- concentration (25 mM) that was used during the pH dependence experiment, since a previous study found that at pH 7.1 (the apparent pK_{a2} in the presence of azide) PSII exhibits an apparent K_m of ~1 mM for Cl^- (*24*).

If we neglect the effects of the OH^- displacement of Cl^-, the shift of both pK_as defining the pH optimum to more neutral values may be interpreted at its simplest using a model in which anion binding influences the pK_as of nearby activating residues (i.e., those involved in catalysis). A similar model was introduced early on by Massey for the activation of fumarase by phosphate (*25*). He proposed that binding of phosphate to a nonactivating basic residue would reduce the dissociation of nearby activating basic and/or acidic residues, thereby shifting the pH maximum upward. This thinking may be applied in the case of the Cl^- activation of PSII. When Cl^- is bound, it probably influences the protonation state of

nearby acidic and basic residues so activity is optimized. N_3^- in place of Cl^- introduces a different charge distribution, since it is long and linear with negative charge delocalized over the three N atoms. If the N_3^- ion were positioned such that it moved negative charge toward the acidic residue but away from the basic residue relative to chloride, the pK_a of the acidic residue would shift upward while that of the basic residue would shift downward. This would result in a narrowing of the pH maximum as observed for the pH dependence curve of O_2 evolution activity studied here. We suggest a mechanism such as this would provide a simple, workable explanation for the mode of inhibition of O_2 evolution by azide.

This paper presents a first look at how the azide anion interacts with the chloride site of photosystem II. As a small anion, azide may be more likely than previously tested amines (other than ammonia) to bind at the chloride site, rather than block the access of chloride to its site. Since it is a nitrogen-based anion, it would provide a convenient probe of the chloride site using magnetic resonance techniques. Thus, future investigation of azide binding to the oxygen-evolving complex may prove to be an attractive area for further research on the mechanism of O_2 production.

ACKNOWLEDGMENT

We thank Prof. James Barborak for assistance in purifying the electron acceptor PPBQ. We also thank a reviewer for an interesting suggestion. We are very appreciative of the technical assistance of Stacy Stinson, Christine Maher, and Thomas Kuntzleman during the various stages of this work.

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BI983075C