Interaction of Ammonia with the Water Splitting Enzyme of Photosystem II†

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ABSTRACT: The effects of NH_3 on the oxygen evolving enzyme have been investigated with EPR and steady-state O_2 evolution. The following results were obtained. At low light intensity O_2 evolution occurs in all centers even though ammonia is bound. This binding occurs in the S_2 state and results in a modification of the multiline signal as reported earlier. However, the oscillations with flash number of the amplitude of the EPR signal are virtually unaffected, indicating that NH_3 binding does not prevent S-state advancement. Inhibition of O_2 evolution by NH_3 measured at light intensities that are nearly saturating for untreated photosystem II is interpreted as being due to a slow down in the rate of S-state cycling. At very high light intensities NH_3 is not able to inhibit oxygen evolution presumably because NH_3 binding is S state dependent and the susceptible S state (S_2) is turned over too quickly. NH_3 binding resulting in the modified multiline signal does not occur in S_1. When S_1 is formed from fully NH_3 modified S_2 by deactivation or by three further flashes, the S_1 state does not have NH_3 bound. NH_3 thus dissociates easily from S_1. Earlier reports of NH_3 binding in S_1 may be explained by the observation that NH_3 binding can occur upon incubation of samples in S_2 at temperatures as low as 198 K. Evidence is obtained for an NH_3 binding occurring slowly (30 s) in S_3. This binding results in a block in S-state advancement as suggested earlier [Velthuys, B. R. (1975) Thesis, University of Leiden]. The results are interpreted in two possible models: (1) NH_3 binding in S_2 occurs in a substrate site, but it is rapidly exchanged by water upon S_4 formation. (2) NH_3 binding in S_2 is not in a substrate site but instead in a structural site and remains bound while water is oxidized. Inherent in this model is that other NH_3 binding sites, i.e., the Cl^- site, and the slow NH_3 binding site in S_3 could be the true substrate sites. Some mechanistic implications are discussed.

Photosystem II catalyzes the light-driven oxidation of water to oxygen and the reduction of plastoquinone, the electron donor to the cyt b_6f complex [see Babcock (1987) for a review]. Absorption of light gives rise to a charge separation resulting in the oxidation of P_{680},[1] the chlorophyll species that acts as the first electron donor and in the reduction of a pheophytin. The electron is rapidly transferred to the bound plastoquinone, Q_A, and then to the exchangeable plastoquinone, Q_B, which is double reduced before it exchanges with an oxidized plastoquinone molecule of the pool. The oxidized primary donor, P_{680}^+, is reduced by tyrosine 161, Tyr_Z, located on the D1 protein (Barry & Babcock, 1987; Debus et al., 1988). Tyr_Z, in turn, is reduced by a cluster of four manganese ions, the structure of which is under debate [see Pecoraro (1988) for a review]. Four consecutive charge separations in the reaction center of PS-II create the oxidizing equivalents that are necessary for the oxidation of two molecules of water to one molecule of oxygen. The donor side of PS-II cycles through five different oxidation states which are denoted S_n, n varying from 0 to 4 according to the model of Kok et al. (1970). Some or all of the four positives charges are stored on the Mn cluster. The two cofactors Ca^{2+} and Cl^- are required for oxygen evolution [Homann (1987, 1989) for reviews].

The S_2 state is paramagnetic and gives rise to two EPR signals. One, the multiline signal, is centered at $g \approx 2$ (Dismukes & Siderer, 1981). The second is centered at $g = 4.1$ (Casey & Sauer, 1984; Zimmermann & Rutherford, 1984). Both signals can be used as probes of the chemistry occurring in the Mn cluster since the shape and extent of each of the signals are sensitive to changes in its local environment, e.g., the presence of cryoprotectants (Zimmermann & Rutherford, 1986), ligand binding, e.g., NH_3 (Beck et al., 1986; Beck & Brudvig, 1986; Andrêasson & Hansson, 1987), and the replacement of Ca^{2+} by Sr^{2+} (Boussac & Rutherford, 1988a).

By using ammonia as an uncoupler of photosynthetic phosphorylation, it was shown that too high a concentration inhibited electron transport (Krogmann et al., 1959). Inhibition occurred on the donor side of PS-II since activity could be restored by the addition of electron donors (Vernon & Zaugg, 1960). The form of ammonia which inhibited oxygen evolution was shown to be the free base, NH_3 (Hind & Whittingham, 1963). Because of the similarity between ammonia and water and because NH_3 is a stronger base than H_2O in Lewis classification, NH_3 has been assumed to be a substrate-like inhibitor of the water splitting enzyme. Luminescence experiments by Zankel (1971) showed that the effect of NH_3 was enhanced after the third and following flashes. Delrieu (1975) found an NH_3-induced increase of the misses in S-state advancement. Ammonia inhibits oxygen evolution by binding to two different sites (Sandusky & Yocum, 1983, 1984, 1986). In one of these sites NH_3 competes

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[1] Abbreviations: P_{680}, reaction center chlorophyll (Chl) of photosystem II (PS-II); Tyr_Z, electron donor to P_{680}; Tyr_D, side-path electron donor of PS-II responsible for EPR signal II_{slow}; Q_A, primary quinone electron acceptor of PS-II; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetate; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; PPBQ, phenyl-*p*-benzoquinone.

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with chloride (Isawa et al., 1969; Sandusky & Yocum, 1983, 1984, 1986). These two sites have been correlated with changes in the S_2 EPR signals (Beck et al., 1986; Andréasson & Hansson 1987).

Binding of ammonia to the chloride site seems to induce a stable $g = 4.1$ signal (Beck & Brudvig, 1986). In samples prepared with sucrose as a cryoprotectant, NH_3 binding to the Cl^- site decreases the width from 40 to 30 mT, and the g value is shifted to 4.2 (Andréasson & Hanssson, 1987). Primary amines and hydroxylamine also bind to the Cl^- site; the latter reduces the S_1 and S_2 states into the S_{-1} and S_0 states, respectively (Bouges-Bocquet, 1973; Beck & Brudvig, 1988a; Sivaraja & Dismukes, 1988). This is probably caused by direct reduction of the Mn cluster by reducing agents, suggesting that the NH_3 binding site and the manganese are in close proximity. Binding of NH_3 in the Cl^- site can occur in the S_1 state but is reported to be more efficient in the S_2 state (Beck & Brudvig, 1986; Andréasson et al., 1988). The inhibition of oxygen evolution by replacement of Cl^- with amines might be expected to be similar to that which occurs when Cl^- is depleted from its site. It has been shown that Cl^- depletion leads to inhibition of S-state advancement after S_2 formation (Theg et al., 1984; Itoh et al., 1984; Ono et al., 1986).

The binding of NH_3 to the other site (the non-chloride site) results in a modification of the multiline signal (Beck & Brudvig, 1986; Beck et al., 1986). This effect is independent of the Cl^- concentration. After binding of NH_3 in the non-chloride site, it has been shown by electron spin echo envelope modulation that the N atom of NH_3 is directly liganded to the Mn cluster (Britt et al., 1989). It is generally assumed that it is the non-chloride NH_3 binding site which is the binding site for substrate water (Velthuys, 1975; Sandusky & Yocum, 1983, 1984, 1986; Beck et al., 1986; Beck & Brudvig, 1986, 1988a,b; Andréassona & Hansson, 1987; Britt et al., 1989). Binding of NH_3 in the non-chloride site has been reported to occur in the S_2 state and not in the S_1 state (Beck et al., 1986; Beck & Brudvig, 1986). However, Ono and Inoue (1988) and Andréasson et al. (1988) have reported that after incubation at sufficiently high concentration, NH_3 can bind in the non-chloride site in the S_1 state.

Velthuys (1975) showed that inhibition of the S-state cycle by NH_3 required two dark incubation times, one short ($\approx$0.3 s) in the S_2 state and one longer ($\approx$1 s) in the S_3 state. After binding had occurred in both ot these states, the $S_3Z^+ \rightarrow S_0$ transition was inhibited. In the work of Velthuys the binding of NH_3 in the S_2 state (i.e., the reaction that is known to induce a modified multiline EPR signal) was reported to modify the stability of the S_2 state in the dark, probably by decreasing its redox potential. This result was recently confirmed by thermoluminescence experiments (Ono & Inoue, 1988). Since a relatively long incubation with NH_3 in the S_3 state is required to inhibit the S-state cycle, it is difficult to reconcile the observations of Velthuys (1975) with the reported inhibition of oxygen evolution by NH_3 in continuous light (Sandusky & Yocum, 1983, 1984, 1986). This difficulty is compounded by the matching of the two inhibitory sites defined by Sandusky and Yocum with the binding sites in S_1 and S_2 in the EPR studies (Beck et al., 1986; Beck & Brudvig, 1986).

In this work we have focused our attention on the relationships between the inhibition of oxygen evolution, the inhibition of the S-state cycle, and the appearance of the modified EPR multiline signal in the presence of NH_3.

Materials and Methods

Photosystem II particles from spinach chloroplasts were prepared according to the method of Berthold et al. (1981) with the modifications of Ford and Evans (1983) and were washed twice and stored at −80 °C in 10 mM NaCl, 2 mM MES, pH 6.5, and 1 mM EDTA at approximately 6 mg of Chl/mL. For low-temperature illumination experiments the samples were put in calibrated quartz EPR tubes, and after 30-min dark adaptation on ice, additions were made to final concentrations of 50 mM HEPES, pH 7.6, and either 100 mM NaCl or 100 mM NH_4Cl. Then PPBQ as an external electron acceptor, dissolved in DMSO, was added to a final concentration of 1 mM. Except when otherwise stated, the dark-adapted samples were incubated for approximately 1–2 min with 100 mM NH_4Cl or 100 mM NaCl before being frozen at 77 K.

The samples were illuminated for $\approx$3 min in a nonsilvered Dewar in a solid CO_2–ethanol bath cooled further to 185–198 K by the addition of liquid nitrogen. Illumination was done with an 800-W projector through $CuSO_4$ and infrared filters. Illumination was done at 185–198 K, as indicated. Samples were cooled to 173 K, in a second ethanol bath, and then ethanol was rapidly removed from the exterior of the tube prior to freezing of the sample in liquid N_2. For flash experiments the samples were diluted to 2.7 of mg Chl/mL in 100 mM NaCl, 2 mM MES, pH 6.5, and 1 mM EDTA. After 30 min in the dark on ice the samples were equilibrated at room temperature and were given one preflash from a Nd-YAG laser (Spectra Physics DCRSG; 8-ns pulse; 300 mJ) and incubated for $\approx$10 min in total darkness at room temperature. Then 50 mM HEPES, pH 7.6, and either 100 mM NaCl or 100 mM NH_4Cl (final concentrations) and 1 mM PPBQ were added. Within 1 min the appropriate sequence of flashes was given. After the flash sequence the samples were transferred (<2 s) to a CO_2–ethanol bath at 198 K and then to 77 K. EPR spectra were recorded at 8 K with a Bruker ESP 300 X-band spectrometer equipped with an Oxford Instrument cryostat. Dark incubations of the samples at temperatures higher than 198 K were done with a homebuilt continuous nitrogen flow system. The temperature at the sample position was measured with a thermocouple. The heating of the sample during low-temperature illumination was less than 1 K as measured directly by a thermocouple. The nitrogen flow was high to minimize the temperature gradient (estimated as $\approx$1–2 K) over the sample.

The contributions of both modified and unmodified multiline signals in the spectra of Figures 3 and 4 were estimated by finding the proportion of the two spectral forms which gave rise to the mixed spectrum by using computer subtraction.

The fast-freeze experiment was done with a System 1000 chemical/freeze quench apparatus (Update Instruments). After dark adaptation in a syringe in a medium containing 50 mM HEPES, pH 7.6, and 100 mM NH_4Cl the sample was pushed into a glass capillary cell ($\approx$35 μL) with one end extended to a very fine jet. In this cell the sample was given a saturating laser flash. A second push ejected the sample through the jet as a fine spray into a funnel connected to an EPR tube, both of which were filled with cooled isopentane (135–140 K). The exact freezing time is difficult to determine, but the time required to empty the cell was <10 ms, and the time needed to reach the isopentane surface was negligible. After several runs the frozen droplets were pushed to the bottom of the EPR tube, and the isopentane was removed.

Oxygen evolution in continuous white light was measured with a Clark type electrode at 23 °C. Samples were diluted to 20 μg of Chl/mL in the indicated media in the presence of 0.5 mM PPBQ. The intensity of light was varied with neutral-density filters. The active form of ammonia is the free

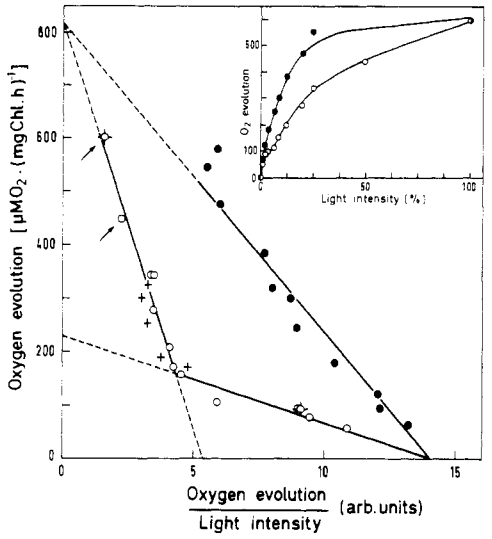

FIGURE 1: Eadie–Hofstee plot of the light-intensity dependence of oxygen evolution in PS-II particles under continuous illumination at 23 °C. The reaction mixture was 10 mM $CaCl_2$, 20 mM HEPES, pH 7.6, 0.5 mM PPBQ, and (●) 100 mM NaCl, (○) 100 mM NH_4Cl, or (+) 200 mM NH_4Cl. The points indicated by arrows were obtained by successive dilutions of the sample. The inset shows the direct plot of the activity versus light intensity for the same data. 100% light intensity is an arbitrary value that gave maximum activity in the sample treated with NH_3.

base NH_3 (Hind & Whittingham, 1963; Velthuys, 1975), which explains the high pH used [$pK_a(NH_4^+/NH_3)$ = 9.24].

RESULTS

The nature of the inhibition of oxygen evolution induced by NH_3 was studied by recording the activity at different light intensities. Under close to saturating light for the control, the activity was ≈590 μmol of O_2/[(mg of Chl)·h] whereas for the same light intensity the presence of 100 mM NH_4Cl decreased the activity to ≈340 μmol of O_2/[(mg of Chl)·h] (inset of Figure 1). Similar inhibition due to NH_3 has been reported earlier (Sandusky & Yocum, 1983; Andréasson et al., 1988). However, Figure 1 shows that the light-intensity dependence of oxygen evolution in PS-II particles under continuous illumination is different in the control compared to that in the presence of NH_4Cl. Maximum activity occurs at a much higher light intensity in the NH_3-containing sample. The maximum activity in the NH_3-containing sample is similar to that in the control. The extrapolated V_{max} at infinite light intensity is the same in both samples. At the lower light intensities, 100 mM NH_4Cl decreased the V_{max}, but all centers were still functioning since both of the straight lines extrapolate to the same value when the intensity is zero. The same x intercept for the curves in Figure 1 reflects an equal relative quantum yield at very low light intensity. Thus, the decrease of V_{max} in the presence of NH_3, extrapolated from the low light intensity part of the curve, could indicate a slowdown of a step in the overall oxygen evolution process. The inability of NH_3 to inhibit oxygen evolution at high light intensities indicates that no NH_3 binding occurs when the S states turnover rapidly. The same results were observed with 200 mM NH_4Cl, indicating that 100 mM NH_4Cl was a saturating concentration at pH 7.6.

EPR measurements were used to investigate the effect of NH_3 on S-state cycling in flashing light. First, however, the effects of ammonia on the EPR signals as described by earlier workers were looked at (Beck et al., 1986; Beck & Brudvig 1986; Andréasson & Hansson, 1987). Figure 2 shows the signal induced by illumination at 188 K in PS-II membranes

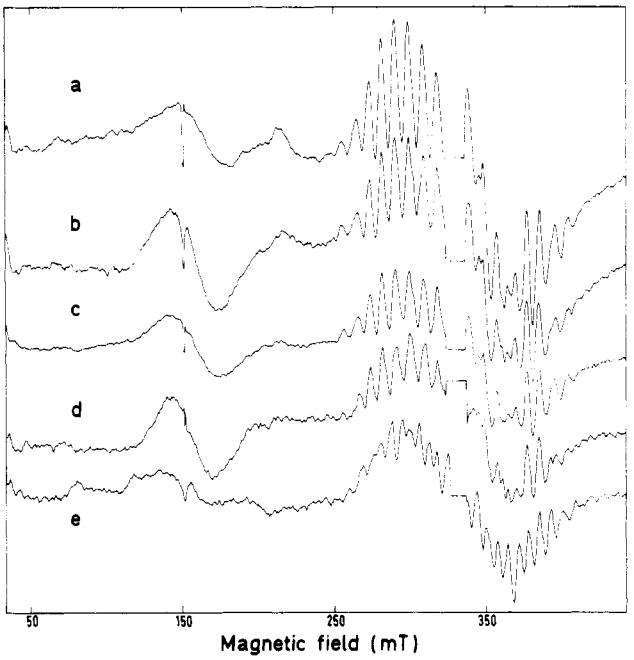

FIGURE 2: Light-induced EPR spectra of PS-II particles. Illumination was done at 188 K after 1–2-min incubation in the dark at pH 7.6 with 100 mM NaCl (a), 100 mM NH_4Cl (b), or 10 mM NH_4Cl (c). Spectrum d corresponds to 60-min incubation in the dark with 100 mM NH_4Cl. Spectrum e is the same sample used for spectrum d but warmed in the dark to 253 K for 30 s followed by cooling to 77 K. Spectra were obtained by subtracting the spectrum recorded before the illumination. Instrument settings: microwave frequency, 9.44 GHz; modulation amplitude, 22 G; temperature, 8 K; microwave power, 20 mW; The Chl concentrations are different in spectra a–d.

at pH 7.6. This temperature was chosen for the reasons explained below. As expected NH_4Cl from 10 (spectrum c) to 100 mM (spectrum b) had no effect on the shape of the multiline signal if compared to the control (spectrum a). Even incubation for 60 min with 100 mM NH_4Cl in the dark at 0 °C prior to low-temperature illumination did not induce any significant change in the shape of the multiline generated. This agrees with Beck and Brudvig (1986) but apparently contrasts with two more recent reports of partially modified multilines under these conditions (see below for a possible explanation of this). To obtain the described transformation of the multiline signal after the binding of NH_3 (Beck et al., 1986; Beck & Brudvig, 1986), the sample with ammonia was warmed to 253 K (spectrum e). This treatment also abolishes the broad line at 350 mT (g = 1.9) in spectra 2a–2d which arises from $Q_A^-Fe^{2+}$ (Rutherford & Zimmermann, 1984), because of its reoxidation by PPBQ.

In these experiments the chloride concentration was kept constant at 100 mM in all samples. Sucrose, which has been suggested to interfere with Cl^- binding (Beck & Brudvig, 1988b), was not present in these samples. The high Cl^- concentration is expected to minimize NH_3 binding to the Cl^- site (Sandusky & Yocum, 1986). Some changes in the g = 4.1 signal can be seen. Spectra b and d (100 mM NH_4Cl) have a small increase in the g = 4.1 signal compared to the control. This is a minor effect relative to the NH_3-induced changes reported at lower Cl^- concentration (Beck & Brudvig, 1986). In addition, a minor shift in its position and a change in its width to 32 mT occur that result from the presence of some 30-mT form at g = 4.2. These effects are due to NH_3 binding in the Cl^- site in some centers as describe earlier (Beck & Brudvig, 1988b; Andréasson et al., 1988), but the high Cl^- concentration is probably responsible for the small scale of these effects.

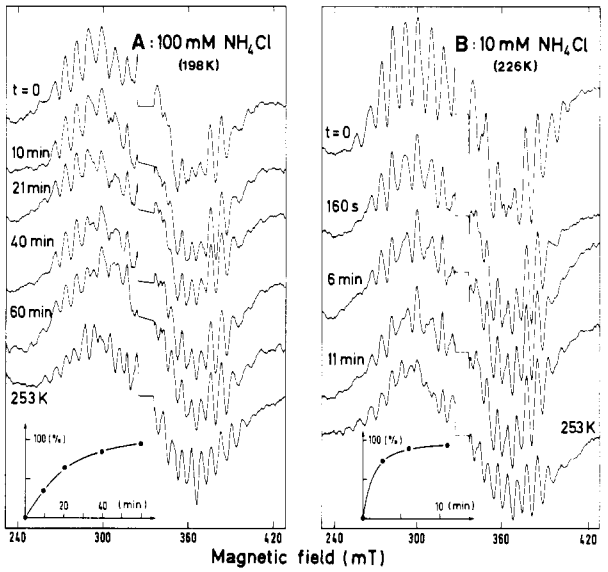

FIGURE 3: (A) EPR difference spectra recorded after a 188 K illumination in the presence of 100 mM NH_4Cl (spectrum $t = 0$) and after dark incubation for various times at 198 K. The spectrum at the bottom of the figure was recorded after dark incubation for 30 s at 253 K followed by a cooling of the sample at 77 K. (B) Same as (A) but in the presence of 10 mM NH_4Cl and 100 mM NaCl. Incubation was performed at 226 K. Instrument settings were as in Figure 2. The insets show the time course of the modification of the signal.

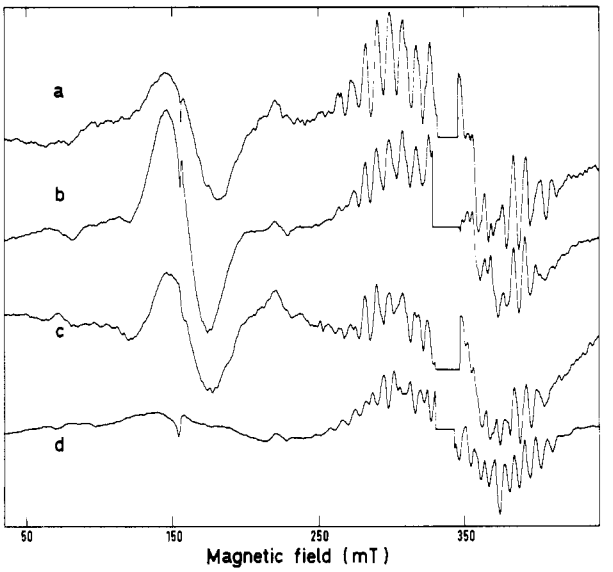

FIGURE 4: Light-induced EPR signals from PS-II-enriched membranes in the presence of 0.3 M sucrose. Illumination was done at 188 K after 1–2-min incubation in the dark at pH 7.6 with 100 mM NaCl (spectrum a) or 100 mM NH_4Cl (spectra b–d). Spectrum b was recorded immediately after illumination. Spectrum c was recorded after a further 15-min dark incubation at 198 K following illumination. Spectrum d was recorded after warming in the dark to 253 K for 30 s followed by cooling to 77 K. Spectra were obtained by subtraction of the corresponding dark spectra before the illumination. Instrument settings were as in Figure 2.

The observation made here and earlier (Beck & Brudvig, 1986), that no modification of the multiline signal occurred upon illumination at low temperature, contrasts with more recent reports by Ono and Inoue (1988) and Andréasson et al., (1988). We looked for an explanation for this discrepancy. We were surprised to find that the normal multiline signal, produced by illumination at 198 K of a dark-adapted sample in the presence of 100 mM NH_4Cl, changed into a modified signal when incubated for relatively short times even at 198 K. Figure 3A shows the time course of this modification at 198 K. The $t_{1/2}$ of the modification was ≈15 min. The temperature dependence for the time course of the modification in the presence of 100 mM NH_4Cl was studied over the temperature range from 198 to 210 K. The Arrhenius plot was linear, and the activation energy was estimated to be ≈70 $kJ \cdot M^{-1}$ (result not shown). This value is only a first approximation since the reaction involves supramolecular complexes. However, its high value is more likely to reflect a binding than a conformational change [Fink and Cartwright (1981) for a review]. Although the extent of the modification in 10 mM NH_4Cl, is smaller, the modification of the signal at low temperature could also be observed at this concentration ($t_{1/2} \approx 1.5$ min at 226 K; Figure 3B). A decrease in the $g = 4.1$ signal is also observed during the incubation. This decrease is probably a charge recombination since we do not observe a parallel increase in the multiline signal and since the signal of Q_A^- disappears [see also Zimmermann and Rutherford (1984, 1986)]. Moreover, the activation energy for the kinetics of the decrease of the $g = 4.1$ signal is independent of ammonia concentration and close to 15 $kJ \cdot M^{-1}$ (not shown).

The experiments in the work of Ono and Inoue (1988) and Andréasson et al. (1988) were done in the presence of sucrose. We have tested, under our conditions, whether incubation in the dark in the presence of 100 mM NH_4Cl and sucrose resulted in a modified multiline signal after low-temperature illumination at 185 K. Figure 4 shows that illumination at 188 K of a sample incubated with NH_3 resulted in a nearly normal multiline signal (spectrum b) if compared to the control (spectrum a). Furthermore, after a 15-min dark incubation at 198 K of an illuminated sucrose sample the extent of the modification of the signal (spectrum c) is similar to that observed in the absence of sucrose (see Figure 3A). Again, warming induced the modification of the signal (spectrum d). The main difference with Figure 2 is the $g = 4.2$ signal with a modified line width of 28 mT. This probably reflects a greater displacement of Cl^- in the Cl^- site (Andréasson et al. 1988) which is enhanced by sucrose (Beck & Brudvig, 1988). After the sample was warmed at 253 K the $g = 4.2$ signal disappears. We should also mention that we observed a decrease in width of the $g = 4.2$ signal in the presence of sucrose and NH_3 after an illumination at 140 K.

The oxygen evolution measurements (Figure 1) suggest that all centers turn over in O_2 evolution at low light intensity in the presence of NH_3. This was tested by EPR measurements in samples excited with saturating laser flashes. Figure 5 shows the spectra obtained with samples frozen after a series of flashes in the presence of 100 mM NaCl (A) or 100 mM NH_4Cl, (B). The EPR signal arising from the S_2 state was modified in the presence of NH_4Cl. Due to the low amplitude of the multiline signal in the presence of NH_3 in the low magnetic field part of the spectrum (see Figure 2e), we chose to record only the high-field part in flash experiments. The amplitude of the multiline signal versus the flash number (Figure 5C) was nearly the same as in the control. Ammonia induced no inhibition of S-state cycling.

We have shown that NH_3 binding can take place even at 198 K (Figure 3). An explanation for the apparent lack of inhibition of S-state turnover in Figure 5 could be that ammonia binding is slow relative to the flash spacing (0.6 s) but can occur during the time required for the cooling of the sample. As a first experimental test of this possibility, we varied the flash spacing from 0.2 to 2.5 s (the maximum spacing without significant deactivation-induced damping) while measuring the amplitude of the signal after the fifth flash. No amplitude variation occurred, indicating no inhib-

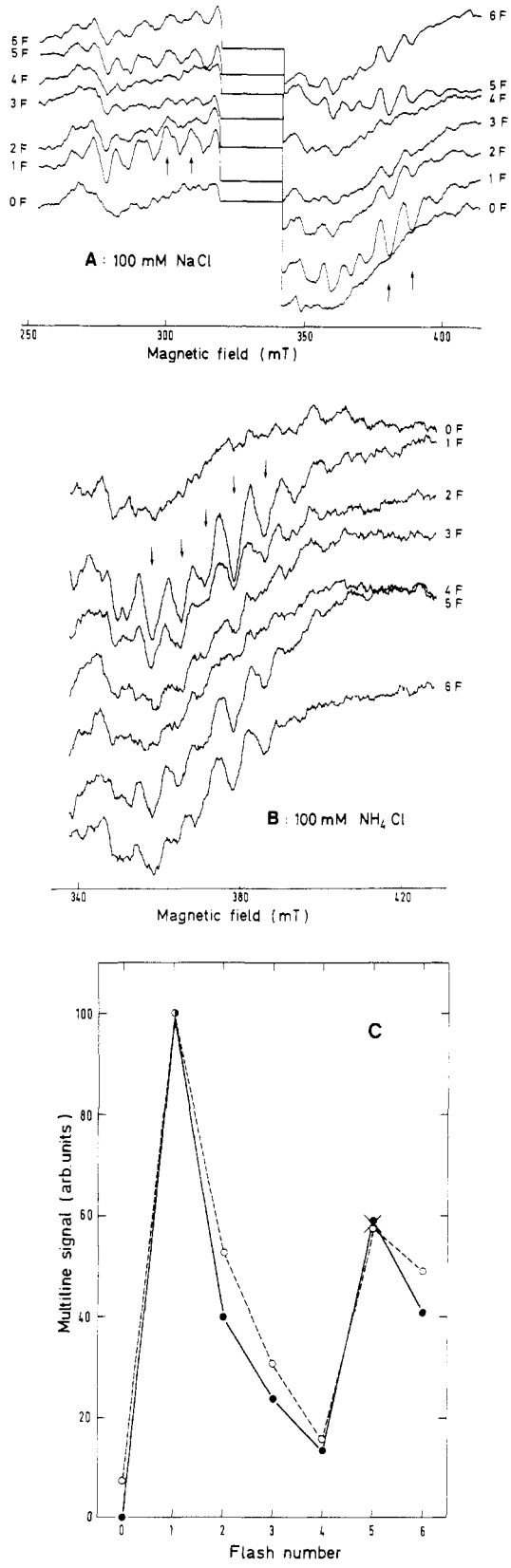

FIGURE 5: (A) EPR spectra after a series of saturating laser flashes given at room temperature to samples in the presence of 100 mM NaCl at pH 7.6. (B) As in (A) with 100 mM NH_4Cl. (C) Amplitude changes of the multiline signal plotted versus the flash number in the presence of 100 mM NaCl (●) or 100 mM NH_4Cl (○). The amplitude of the signal was estimated from the added height of the peaks marked with arrows in (A) and (B). The flash spacing was 0.6 s. The cross (×) corresponds to the amplitude of the multiline signal after five flashes in a flash series with a flash spacing of 0.2 or 2.5 s in the presence of 100 mM NH_4Cl. Instrument settings were as in Figure 2.

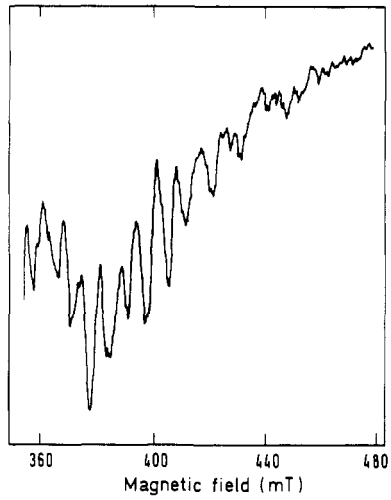

FIGURE 6: EPR spectrum recorded after one flash at room temperature and rapid freezing in an isopentane bath at 135–140 K. The sample was incubated for 30 min in the dark at room temperature in 100 mM NH_4Cl and 50 mM HEPES, pH 7.6, prior to flash illumination. The estimated freezing time was ≪100 ms. The spectrum was recorded on a Bruker ESR 200 D X-band spectrometer. Instrument settings were as in Figure 2.

ition. As a second experimental test EPR samples in the presence of NH_3 were cooled to 140 K rapidly after the flash. At the fastest freezing time, the signal appeared modified (Figure 6). Although there are some difficult experimental questions in determining the actual time of cooling, we estimate that it takes much less than 1 s. From O_2 evolution measurements (see Discussion) and Figure 1, we estimate the time for ammonia binding to S_2 in all centers to be less than 80 ms. On the basis of an activation energy of 70 $kJ \cdot M^{-1}$, for the binding of NH_3, the extrapolation to 296 K gives also a time of binding in the millisecond range. It is concluded then that NH_3 binding to S_2 does occur rapidly and that it has little effect on S-state advancement. We only observe a slight increase of the misses in the presence of NH_4Cl. We should mention that in dark-adapted membranes we observed a small decrease of the amplitude of signal II_{slow} at pH 7.6 (with or without NH_3) which increases the misses on the first flash ($S_1D \rightarrow S_1D^+$). We present no calculation of the variation in the miss parameter in the experiment in Figure 5 since the stability of signal II_{slow} is not known under our conditions.

In agreement with earlier suggestions (Velthuys, 1975), we have observed with EPR that binding of NH_3 in the S_2 state results in an increase in the deactivation time of the S_2 state ($t_{1/2} \approx 10$ min; not shown but see Figure 7) and as proposed earlier may reflect a decrease in the redox potential of S_2 (Ono & Inoue, 1988; Andréasson et al., 1988). Nevertheless, in some preparations a faster phase ($t_{1/2} \approx 3$–5 min), similar to that in untreated membranes, could be observed (not shown). In contrast, Figure 7 shows that the S_3 state formed in the presence of 100 mM NH_4Cl and PPBQ is deactivated faster ($t_{1/2} \approx 1$ min) than that in the control membranes at pH 6.5 ($t_{1/2} = 5$ min) (Styring & Rutherford, 1988).

Ten-minute incubation following two flashes results in deactivation of S_3, forming the S_1 state in a part of the centers (Figure 7). Further illumination of this sample at 198 K induced a normal multiline signal. This results shows that the S_2 to S_1 deactivation results in debinding of NH_3. Figure 8 shows the signal induced by illumination at 198 K of a sample preilluminated by four flashes in the presence of 100 mM NH_4Cl (spectrum a). The signal is similar to that observed in the control (spectrum b) and does not correspond to the modified signal induced by one flash (spectrum c). This in-

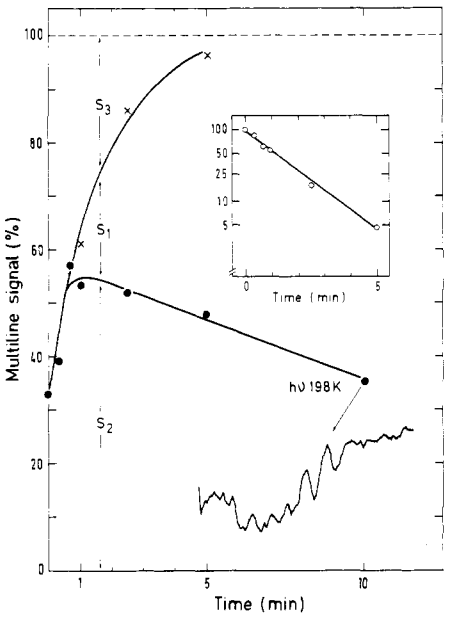

FIGURE 7: Stability of the S_3 state in the presence of 1 mM PPBQ and 100 mM NH_4Cl at pH 7.6 with synchronized S states. The samples were given two flashes and then dark incubated at room temperature for various times. The multiline signal was measured (●). Then the samples were illuminated at 198 K, and the multiline signal was measured again. The curve (×) results from the addition of the extra signal induced at 198 K to that of the modified signal recorded after deactivation following the two flashes. The curve (×) gives the increase of $S_1 + S_2$ versus time and corresponds to the reduction kinetics of S_3 [see Styring and Rutherford (1988)]. The multiline signal induced by illumination at 198 K is unmodified. Therefore, we have normalized its amplitude to that of the modified signal. For that, we have divided the amplitude of the extra signal induced by 198 K illumination by the amplitude of the signal induced at 198 K in the zero flash sample. The ratio obtained for each deactivation time was multiplied by the amplitude of the modified signal induced by one flash at room temperature. Instrument settings were as in Figure 2. The inset shows a logarithmic plot of the time course for the deactivation of the S_3 state. The arrow indicates the 198 K light-induced signal after 10-min deactivation.

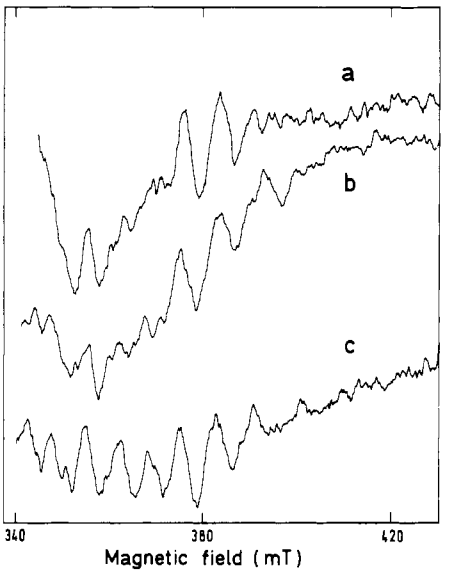

FIGURE 8: EPR spectrum corresponding to the multiline signal induced by illumination at 198 K of a sample given four flashes at room temperature in the presence of 100 mM NH_4Cl (a) or 100 mM NaCl (b). Spectrum c shows the light minus dark multiline signal formed after one flash in the presence of 100 mM NH_4Cl. Instrument settings were as in Figure 2.

dicates that NH_3 binding in S_1 does not occur when S_1 is formed from modified S_2 by three further flashes.

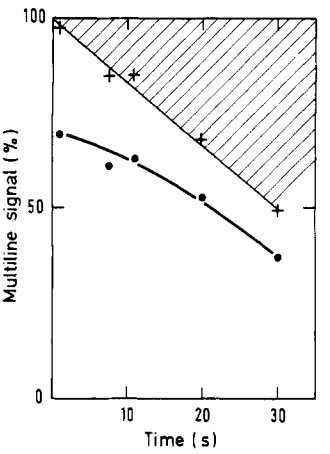

FIGURE 9: (●) Modified multiline signal induced by five flashes versus the time between the second and third flash in the presence of 100 mM NH_4Cl and 1 mM PPBQ. The time between the other laser flashes was kept at 0.6 s. The amplitude of the modified multiline signal is plotted as a percentage of the modified multiline signal formed after one flash, at room temperature, in the presence of 100 mM NH_4Cl. The signal recorded after one flash corresponds to 75% to that recorded after one flash followed by 198 K illumination. (+) Combined amplitude of modified and unmodified multiline signals present after 198 K illumination of samples preilluminated by five flashes. The amplitude of the unmodified multiline signal is calculated relative to the signal induced by 198 K illumination of a dark-adapted sample. Since 198 K illumination gives an ≈35% higher multiline signal compared to that seen after one flash, this factor is taken into account in this plot. The increase of the hatched area reflects the kinetics for the inhibition of the S_3 to S_0 transition. Instrument settings were as in Figure 2.

An inhibition of the S-state turnover was reported to occur only after the binding of a second NH_3 molecule in the S_3 state (Velthuys, 1975). The half-time for this binding in thylakoid membrane was nearly 1 s when the first NH_3 molecule was already bound (Velthuys, 1975). To detect such an effect of NH_3 in the S_3 state, we varied the time between the second and the third flashes in a flash sequence. Then we recorded the amplitude of the multiline signal after the fifth flash (Figure 9). The decrease of the signal when time increases [curve (●)], reflects both the putative inhibition of the S_3 to S_0 transition and the deactivation of the S_3 state. The fast deactivation of the S_3 state to the S_2 state results in PS-II centers in the S_1 state after the fifth flash. Due to the stability of the S_2 state, the fraction of the centers deactivated to the S_1 state after short times (<30 s) is negligible. Therefore, after the fifth flash, an illumination at 198 K was given to each sample to estimate the proportion of centers in the S_2 state formed by deactivation of S_3. The addition of this extra light-induced signal at 198 K to the flash-induced signal [curve (+) in Figure 9] gives the time course for inhibition in the S_3 state (dashed area). For 0.6 s close to 100% of the centers reaching the S_2 state after one flash are in the S_1 or S_2 states after the fifth flash. For a spacing between the second and third flashes of 30 s nearly 50% of the centers are unable to reach the S_1 or S_2 states on the fifth flash and are probably blocked at the S_3 to S_0 transition. This inhibition ($t_{1/2} \approx 30$ s) could be due to the binding of a second NH_3 molecule to the S_3 state, as first proposed by Velthuys (1975).

Discussion

The results of Figure 1 demonstrate a complex relationship between light intensity and oxygen evolution in PS-II membranes in the presence of NH_3. At very high light intensity and at very low light intensity, O_2 evolution activity is virtually unaffected, while between these two extremes inhibition occurs. Previous reports of ammonia inhibition of O_2 evolution have

been made only at light intensities that are saturating for O_2 evolution in control samples; this turns out to be nonsaturating for ammonia-treated material. These results might be explained as follows: (1) Since ammonia binding is S state dependent, the susceptible S state(s) is (are) turned over so rapidly at very high light intensities that ammonia binding and thus inhibition cannot take place. (2) At very low light intensities ammonia binds but does not lead to a block of S-state cycling. Since the light intensity is rate limiting no inhibition is observed. (3) At intermediate light intensities ammonia binding occurs resulting in a slowdown in S-state cycling which is observed as a decrease in O_2 evolution compared to the control.

By extrapolating to the V_{max} in the control sample the time required for one S-state cycle to be completed can be estimated to be ≈20 ms, on the basis of ≈220 Chl/PS-II (i.e., average of 5 ms per S state). The same calculation can be made for the inhibited rate of O_2 evolution in the presence of NH_3 from extrapolation of the linear part of the plot at low light intensities. A value of ≈80 ms per S-state turnover is obtained; this is attributed to a new rate-limiting step on the donor side of PS-II induced by NH_3 binding. Velthuys (1975) has already shown that NH_3 binding in thylakoids results in a slowdown of the reaction $Z^+S_2(NH_3) \rightarrow ZS_3(NH_3)$ from $t_{1/2}$ = 0.4 ms to $t_{1/2}$ = 13 ms. Since this is a $t_{1/2}$ value and since our estimate for the rate-limiting step of 80 ms is the time required to complete the S-state cycle, it is feasible that the slowdown of the S_2- to S_3-state step is sufficient to account for the slow cycling time. However, it is quite possible that the kinetics of other S-state transitions are also affected by ammonia binding. It is also of note that NH_3 binding results in the slowing down of Tyr_Z^+ rereduction as detected by EPR (Yocum & Babcock, 1981). Interestingly, Tyr_Z^+ is only detectable in the tens of milliseconds time scale in approximately 30% of the centers, indicating that rapid donation from the S states to Tyr_Z^+ takes places in the majority of centers (Ghanotakis et al., 1983). It is possible that this represents a slowdown of S-state donation in only one of the four S states. It is significant that the lifetime of Tyr_Z^+ detectable in the millisecond range showed an important 40-ms phase, a value which falls between our estimated rate-limiting S-state cycle turnover time (80 ms) (Figure 1) and the measured $Z^+S_2(NH_3) \rightarrow ZS_3(NH_3)$ half-time (13 ms) (Velthuys, 1975).

From Figure 1 it is concluded that at very high light intensities NH_3 does not have time to bind to the susceptible S state. The extrapolated V_{max} of O_2 evolution activity in the presence of ammonia is the same as that in the control. Thus the S-state turnover time of 20 ms is a rate which does not allow significant ammonia binding to take place. Velthuys (1975) has shown that in tens of milliseconds no NH_3 binding occurred in S_2. At a cycling time lower than this, ammonia binding must occur, giving rise to inhibition in O_2 evolution relative to the control. The results then indicate that the ammonia binding giving rise to the slowdown of O_2 evolution takes place between 20 and 80 ms to be complete. Velthuys (1975) estimated the NH_3 binding rate in S_2 to be 300 ms in thylakoids. The significance of this difference is not clear. Trivial experimental differences could be responsible: (1) The use of thylakoids, which may be more acidic in the closed vesicular space, could result in a lower NH_3 concentration in the space adjacent to the Mn cluster. (2) The use of detergent-isolated PS-II membranes may expose the Mn cluster to more rapid ligand exchange. (3) The presence of sucrose, which is known to affect Cl^- binding (Beck & Brudvig, 1988b) and Ca^{2+} binding (Boussac & Rutherford, 1988b) may also have an influence on NH_3 binding.

Extrapolation to zero light intensity in Figure 1 indicates that no inhibition by ammonia is present when light intensity is limiting. This conclusion is supported by the observation that the oscillation pattern in the NH_3-modified multiline after a series of flashes is virtually identical with that in the control. These results are in contradiction to earlier luminescence (Velthuys, 1975) and thermoluminescence (Ono & Inoue, 1988) results which showed marked NH_3-induced inhibition of S-state advancement in flashing light. Thus we made efforts to rule out the trivial explanation that ammonia binding was slow relative to the flash spacing, thus allowing normal cycling, but that NH_3 binding occurred during cooling. Velthuys (1975) and Ono and Inoue (1988) showed that inhibition of S-state advancement occurred with flashes separated by 1 s. We obtained identical unhibited flash patterns with flash spacings of 0.6 and 1 s (not shown). In addition we measured the signal after the fifth flash using flash spacing ranging from 0.2 to 2.5 s and found no variations (Figure 5). We also made samples frozen to 140 K very rapidly after the flash and found NH_3-induced modifications of the EPR signal at the fastest freezing times (Figure 6). In addition subsecond binding times for NH_3 with S_2 are predicted from our study of binding rate versus temperature. By extrapolation on the basis of an activation energy of 70 $kJ \cdot M^{-1}$, we estimate that NH_3 binding takes 0.5 s at 240 K. Although extrapolation to room temperature is probably not justified, it seems unlikely that the binding will get any slower than this at higher temperatures.

We conclude then that the EPR flash experiment is interpretable as indicating no inhibition of S-state cycling when NH_3 is bound in the site associated with the modification of the multiline signal and that this fits with the interpretation of the O_2 evolution measurements at low light intensity.

What then is the origin of the discrepancy between these results and those from the luminescence and thermoluminescence (Velthuys, 1975; Ono & Inoue, 1988)? Velthuys (1975) reported that a second ammonia must be bound in S_3 before inhibition of the S_3 to S_0 transition could take place. The binding time ($t_{1/2}$) for this was estimated to be 1 s. We looked for this effect and indeed found evidence that could indicate that binding of NH_3 in S_3 was required before S-state cycling at the S_3 to S_0 level was blocked. In contrast to Velthuys' (1975) measurements, our estimate of the binding time ($t_{1/2}$) was 30 s (Figure 9). Such a long binding time for this event could explain the absence of inhibition of S-state cycling in our experiments. However, we have yet to explain the large discrepancy in the measured NH_3 binding time in S_3. Again we must resort to experimental conditions. Both Velthuys (1975) and Ono and Inoue (1988) had high concentrations of sucrose in their experiments, which is thought to perturb Cl^- binding phenomena (Beck & Brudvig, 1988b). It certainly could affect NH_3 binding to this site, particularly if the S_3 site is a Cl^- binding site.

Evidence is presented which indicates that NH_3 binding of the type which results in modification of the multiline EPR signal only occurs in S_2. Formation of unmodified multiline signals by low-temperature illumination occurs prior to binding of ammonia, which results in the modifications. This agrees with the original conclusions of Velthuys (1975) and the EPR work of Beck et al. (1986) and Beck and Brudvig (1986). A significant difference to the earlier work is our observation that the modification of the multiline signal can occur without thawing the sample. Indeed, incubation for a few minutes at 198 K is all that is required for complete modification to occur (Figure 3). We suggest that this unexpected low-temperature

binding effect can explain the recent reports of Ono and Inoue (1988) and Andréasson et al. (1988), who found partially modified multiline signals after low-temperature illumination and interpreted this as being due to NH_3 binding to the S_1 state.

It has been generally accepted that experiments carried out at temperatures around 200 K do not allow ligand exchange reactions in PS-II oxygen evolving enzyme (Beck et al., 1986; Beck & Brudvig, 1986; Ono & Inoue, 1988; Andréasson et al., 1988). The results in this paper clearly show that this assumption is not justified.

S_1 formed from NH_3-modified S_2 either by back-reaction or by three further flashes does not have ammonia bound to the site which gives the modified multiline signal (Figures 7 and 8). This indicates that debinding of NH_3 occurs prior to, or upon, S_1 formation. Thus, ammonia binding in this site on S_1 is not rate limited, and indeed, incubations in the dark resulted in no appearance of modified multiline when photoinduced at 188 K.

In agreement with earlier work (Velthuys, 1975; Delrieu, 1975; Beck & Brudvig, 1986; Ono & Inoue, 1988), we observed that the modified S_2 state was more stable at room temperature compared to untreated S_2 state. This has been attributed to a shifting of the redox potential to a lower potential (Ono & Inoue, 1988). In addition we have found that the S_3 state formed from NH_3-modified S_2 has a shortened lifetime.

NH_3 binding in S_2 is generally regarded as occurring in a site which is occupied by a substrate water molecule. Here we report that this binding results in no inhibition of S-state turnover. Instead, it is suggested that the kinetics of S-state turnover are slowed down. A block in S-state turnover only occurs when a second event, presumably the binding of a second NH_3 molecule, occurs in a slow reaction in S_3. Although these results seem surprising, they are largely in accordance with the early work of Velthuys (1975).

If NH_3 binding in S_2 is indeed to a substrate site, then the lack of inhibition of S-state cycling implies that the bound NH_3 is rapidly exchanged for the usual substrate, H_2O, upon formation of S_4. This rapid exchange process presumably can not occur when a second NH_3 molecule (bound in S_3) is present, resulting in inhibition of the S_3 to S_0 transition. An alternative interpretation is that the NH_3 binding site in S_2 is *not* a substrate site. In this case NH_3 could remain bound throughout the water splitting steps, although it may debind in S_0 or S_1 due to the predicted weaker Lewis acidity of the Mn cluster in these more reduced states. It is of note that the prefered interpretation of NH_3 binding in S_2 from the ESEEM results is a bridged Mn–N–Mn structure (Britt et al., 1989). This could represent a purely structural, nonsubstrate binding, site in which a μ-oxo bridge is replaced by a μ-imino bridge by displacement of the oxygen. If NH_3 binding to S_2 is not to a substrate site, it is reasonable to suggest that the other NH_3 binding sites might represent the true water, substrate, sites. The NH_3/Cl^- binding site in S_1 and the slow NH_3 binding site in S_3 might be the true substrate sites.[2] That Cl^- occupies the substrate site in lower S states could have mechanistic significance in that it may prevent water oxidation in too low S states (e.g., S_1–S_3) (Rutherford, 1989; Thompson, 1989). This would be desirable since unstable, overreduced, states of the Mn would not be allowed to occur (Rutherford, 1989). In this model also rapid replacement of Mn ligands, this time Cl^-, by H_2O must be involved in the higher S states [see also Radmer and Ollinger (1986)].

[2] It has been reported that the affinity of the Cl^--dependent site for NH_3 in S_1 increases in S_2 (Beck & Brudvig, 1986; Andréasson et al., 1988). Thus, even with the high Cl^- concentration used in the present work, NH_3 binding to the Cl^- site may occur in S_2. Although we have no experimental method of monitoring this putative binding, we must consider that it could contribute to the phenomena reported here. If the Cl^- site is a substrate site, the NH_3 in this site must be replaced by H_2O, at a higher S state. Binding of NH_3 at this site may only become inhibitory when binding of a second NH_3 occurs, i.e., the slow event occurring in S_3. Future experimental work should address this question.

Acknowledgments

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Registry No. NH_3, 7664-41-7; O_2, 7782-44-7; oxidase, water (photosynthetic), 114514-26-0.

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Kinetics of Calcium Channel Opening by Inositol 1,4,5-Trisphosphate†

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ABSTRACT: The subsecond mobilization of intracellular Ca^{2+} by IP_3 was measured with rapid mixing techniques to determine how cells achieve rapid rises in cytosolic [Ca^{2+}] during receptor-triggered calcium spiking. In permeabilized rat basophilic leukemia cells at 11 °C, more than 80% of the 0.7 fmol of Ca^{2+}/cell sequestered by the ATP-driven pump could be released by IP_3. Half of the stored Ca^{2+} was released within 200 ms after addition of saturating (1 μM) IP_3. The flux rate was half-maximal at 120 nM IP_3. Ca^{2+} release from fully loaded stores was highly cooperative; the Hill coefficient over the 2-40 nM range was greater than 3. The delay time of channel opening was inversely proportional to [IP_3], increasing from 150 ms at 100 nM IP_3 to 1 s at 15 nM, indicating that the rate-limiting step in channel opening is IP_3 binding. Multiple binding steps are required to account for the observed delay and nonexponential character of channel opening. A simple model is proposed in which the binding of four IP_3 molecules to identical and independent sites leads to channel opening. The model agrees well with the data for K_D = 18 nM, $k_{on} = 1.2 \times 10^8$ M^{-1} s^{-1}, and k_{off} = 2.2 s^{-1}. The ~1-s exchange time of bound IP_3 indicates that the channel gating sites are distinct from binding sites having ~100-s exchange times that were previously found with radiolabeled IP_3. The ~1-s response time of [Ca^{2+}] to a rapid increase in IP_3 level can account for observed rise times of calcium spikes.

Many hormones, neurotransmitters, and other extracellular stimuli activate the phosphoinositide cascade by binding to cell surface receptors that are coupled to phospholipase C. The subsequent increase in inositol 1,4,5-trisphosphate (IP_3) leads to Ca^{2+} release from intracellular stores (Berridge, 1987; Carafoli, 1987; Putney, 1987). Recent studies indicate that receptor-triggered Ca^{2+} release is often pulsatile rather than sustained. Activated cells exhibit repetitive transient increases in cytosolic Ca^{2+} (Woods et al., 1986; Miyazaki et al., 1986; Jacob et al., 1988). A mechanistic understanding of Ca^{2+} spiking requires detailed knowledge of the kinetics of IP_3-induced Ca^{2+} release. In previous studies with permeabilized rat basophilic leukemia cells (RBL cells), we found that the IP_3-gated Ca^{2+} channel is cooperatively opened by IP_3 with a Hill coefficient of 2.7 (Meyer et al., 1988). Because the time resolution of our earlier study was 4 s, the kinetics of release under physiological IP_3 conditions could not be measured. We report here rapid-kinetic studies of IP_3-induced Ca^{2+} release having a time resolution of 10 ms. The observed dependence of release kinetics on nanomolar IP_3 concentration in the

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