*Biochimica et Biophysica Acta*, 936 (1988) 351–360
Elsevier



BBA 42883

# The interaction of ammonia with the photosynthetic oxygen-evolving system

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(Received 6 June 1988)

Key words: Oxygen evolution; Photosystem II; Manganese; ESR; Ammonia

**The reaction of ammonia with the oxygen-evolving system was investigated using EPR. Two sites with distinct binding properties were found. One site, previously known to be responsible for the modification by ammonia of the multiline EPR signal from the $S_2$ state and believed to be accessible in this state only, was found to bind ammonia also in the $S_1$ state although weaker. The second binding site, identified by the effect of bound ammonia on the shape and position of the $g = 4.1$ EPR signal, was also found to be accessible in both the $S_1$ and $S_2$ states. The apparent dissociation constants for ammonia at the two sites in the $S_1$ and $S_2$ states were determined. In neither state did the binding the ammonia account for the observed inhibition of oxygen evolution, suggesting that binding to other S states plays an important role in the inhibition. Chloride, which is known to interfere with ammonia-induced inhibition of oxygen evolution, was found to compete with ammonia at the site associated with the modification of the $g = 4.1$ EPR signal. The broadening of the hyperfine lines of the multiline EPR signal, seen in the presence of $^{17}O$-labeled water, was still observed after the modification of the signal by ammonia. This indicates that ammonia has not completely displaced water bound to the catalytic site in the $S_2$ state. The results of the binding studies are interpreted in terms of a two state – two site model, where the two states are identified by their EPR signals, the multiline and the $g = 4.1$ signal, respectively, and the two sites identified by the effects of ammonia on these signals and where the equilibrium between the two states is regulated by the binding of ligands to the sites.**

## Introduction

The oxygen-evolving system in PS II of plants and cyanobacteria, which catalyzes the oxidation of water to molecular oxygen, depends for its function on a complex consisting of four manganese ions. This complex serves as a device for the accumulation of positive equivalents which are eventually neutralized by electrons from water (for some recent articles reviewing the role of manganese in photosynthesis, see Refs. 1–3). The successive removal of single electrons from the manganese-containing complex, which advances the oxygen-evolving complex cyclically through the states $S_0$ to $S_4$ with oxygen release on the $S_3$ to $S_0$ transition, is expected to result in oxidation state changes in the manganese and the formation of paramagnetic states. Oxidation state changes were originally suggested from optical measure-

Abbreviations: Mes, 4-morpholineethanesulfonic acid; DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea; EPR, electron paramagnetic resonance; PS, Photosystem; XAES, X-ray absorption edge spectroscopy.

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ments in the ultraviolet [4,5] and have later received support from various other methods, including EPR [6,7], XAES [8], spin-lattice relaxation measurements involving bulk water [9] or EPR Signal $II_{slow}$ [10]. The only paramagnetic state of the oxygen-evolving complex studied closely so far by EPR, $S_2$, is characterized by a complex 'multiline' EPR spectrum centered around $g = 2$ [6,11,12], originating from a mixed-valence cluster of manganese ions. A second population of PS-II centers gives rise to a different EPR signal in the $S_2$ state, centered around $g = 4.1$ [13,14]. XAES measurements suggest that this signal also originates from manganese [15].

Different models have been put forward to explain the existence and properties of these signals. In one of these, both signals are proposed to arise due to different metal–metal interactions in one and the same tetrameric manganese cluster [16]. In a second model, the multiline and the $g = 4.1$ EPR signals are suggested to arise from a mixed-valence pair of manganese ions and an isolated Mn(IV) ion, respectively [17]. Both models include an equilibrium between the two states responsible for the signals; a conformational equilibrium favoring one or another set of interactions in the former, or a redox equilibrium in the latter, placing the unpaired electron in the $S_2$ state on the manganese pair or on the isolated manganese ion.

The two EPR signals have proven to be valuable tools in the study of interactions of the oxygen-evolving complex with agents which interfere with the oxygen-evolving process. Ammonia, which inhibits oxygen evolution, has been shown to bind to the $S_2$ state modifying the multiline EPR signal [18]. There is evidence from experiments using $^{17}O$-labeled water that substrate water may bind to the manganese responsible for this signal [19]. Thus, one reason for the inhibition may be that ammonia excludes water from the catalytic site. However, as yet, there is no firm support for a direct coordination of $NH_3$ to manganese. In fact, such a view has been challenged recently by the observation that $Sr^{2+}$, probably by a conformational effect, induces changes in the multiline signal similar to those of ammonia [20]. In the present work, binding of ammonia to a site does not necessarily imply a direct involvement of manganese.

Ammonia also appears to be able to bind to the $S_1$ state but to a second site, causing a shift in intensity from the multiline to the $g = 4.1$ signal [21]. This site may be one of the two kinetically distinguishable inhibitory binding sites and that to which ammonia binds in competition with chloride [22,23], as has been suggested recently [21,24,25].

Anions, other than chloride, such as $F^-$ and $NO_3^-$, also seem to bind at this site favoring the formation of the $g = 4.1$ EPR signal [24,26].

In this communication we present a further characterization of the effects of ammonia on the oxygen-evolving complex, with a special reference to the properties of the two binding sites in the $S_1$ and $S_2$ states and the relation of the binding to the inhibition of oxygen evolution. Our studies allow us to extend the previously presented models of the binding of ligands to the oxygen-evolving complex and to specify how the binding is affected by the oxidation state of the manganese. A preliminary account of some of the results presented herein was given at the 7th International Congress on Photosynthesis [25].

## Materials and Methods

*Sample preparation*

PS-II-enriched thylakoid membranes from spinach were prepared as described in Ref. 27 and suspended in 20 mM Mes-NaOH (pH 6.3)/15 mM NaCl/5 mM $MgCl_2$/400 mM sucrose. The PS II membranes were stored at about 10 mg chlorophyll/ml at 77 K until used.

PS II membranes were washed with buffer (20 mM Hepes (pH 7.5)/15 mM NaCl/300 mM sucrose), resuspended in the same buffer at about 6–8 mg chlorophyll/ml and transferred to EPR tubes. The sample tubes were dark-adapted on ice for about 2 h before further treatment.

For the reaction with the $S_1$ state, ammonium sulfate was added in darkness to dark-adapted PS II membranes in EPR tubes and allowed to react for 1 min before freezing the sample tubes in an ethanol/solid $CO_2$ bath. The pH of the ammonium sulfate solution was adjusted to that of the buffer used in each experiment. The $S_2$ state was generated by illumination of the samples with white light at 2000 $W/m^2$ for 5 min at 200 K.

For the reaction of ammonia with the $S_2$ state, dark-adapted PS II membranes at about 2 mg chlorophyll/ml in EPR tubes were illuminated at 273 K with a saturating flash from a dye laser and allowed to react with ammonium sulfate in the dark for 15 s before freezing at 200 K. Alternatively, the ammonia-modified $S_1$ state was first generated as outlined above, followed by illumination at 200 K to generate the $S_2$ state. The samples were then thawed in the dark for 40 s to allow the $S_2$ state to equilibrate with ammonia before freezing to stop the reaction.

PS II membranes in an $H_2{}^{17}O$-enriched medium were prepared as described in Ref. 19. The binding of ammonia to the $S_2$ state in these samples was accomplished by illumination at 273 K for 30 s in the presence of 80 $\mu$M DCMU and ammonium sulfate.

*Activity measurements*

The oxygen-evolving activity of the PS II membranes in the final suspension medium, measured at 25 °C with a Clark-type oxygen electrode, was typically 600–700 $\mu$mol $O_2$/mg chlorophyll per h.

*EPR measurements*

EPR measurements were performed as described in Ref. 19 with a Bruker ER 200D-SRC instrument equipped with an Oxford Instruments ESR-9 helium-flow cryostat.

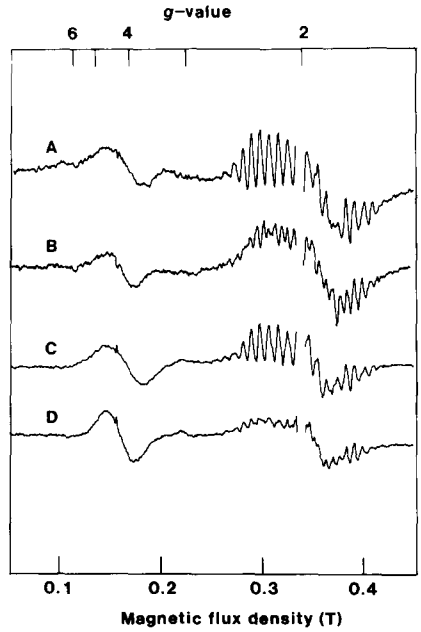



Fig. 1. Effect of $NH_3$ on the EPR signals from the $S_2$ state. (A) dark-adapted PS II membranes, 10 mg chlorophyll/ml in 20 mM Mes (pH 6.3)/15 mM NaCl/400 mM sucrose with 80 $\mu$M DCMU (added dissolved in dimethyl sulfoxide), illuminated for 30 s before freezing. (B) as (A), but with 250 mM $(NH_4)_2SO_4$ added during the illumination. (C) dark-adapted PS II membranes in 20 mM Hepes (pH 7.5)/15 mM NaCl/300 mM sucrose/100 $\mu$M DCMU, after 5 min illumination at 200 K. (D) as (C), but incubated in the dark with 55 mM $(NH_4)_2SO_4$ for 1 min before freezing. The figure shows the light-induced difference spectra. Microwave frequency, 9.46 GHz; power, 20 mW; modulation amplitude, 2.5 mT; temperature, 11 K.

## Results

*Effect of $NH_3$ on the $S_2$-state EPR signals*

PS II membranes, illuminated in the presence of DCMU at 273 K, exhibited both the multiline and the $g = 4.1$ EPR signals. When the illumination was carried out in the presence of $NH_4)_2SO_4$ the characteristic crowding of the hyperfine lines of the multiline signal was evident (Fig. 1A, B) which confirmed the binding of ammonia to the $S_2$ state of the oxygen-evolving complex. However, the $g = 4.1$ EPR signal also was affected by the presence of ammonia. The width of the signal decreased from 36 to 28 mT and the $g$-value shifted to 4.2 (Fig. 1A, B).

*The binding of $NH_3$ to the $S_1$ state*

To see if the $S_1$ state was also capable of binding ammonia, dark-adapted PS II membranes were treated with $(NH_4)_2SO_4$ in the dark, then frozen and subsequently illuminated at 200 K to generate the $S_2$ state. With the usual assumption that ligand-exchange reactions are unlikely to occur in the frozen state, all modifications of the $S_2$ state appearing under these conditions should reflect interactions of ammonia with the $S_1$ state before freezing. Earlier EPR studies have suggested that binding to the site affecting the multiline signal could occur only in the $S_2$ state. However, effects similar to those seen after the binding of ammonia to the $S_2$ state were also evident when the $S_1$ state was exposed to ammonia, i.e., a crowding of the hyperfine lines of the multiline signal, with a decrease in the mean separation between the lines from 9 to 7 mT, and a narrow-

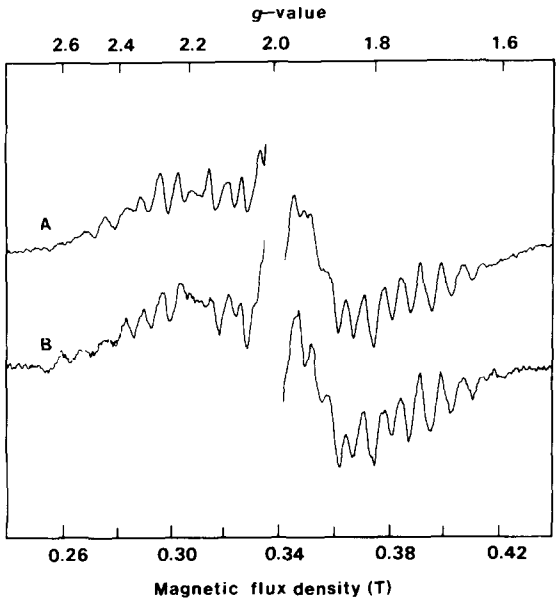



Fig. 2. Effect on the multiline EPR signal after binding to $NH_3$ to the $S_2$ (A) and $S_1$ (B) states, respectively. (A) dark-adapted PS II membranes, 8 mg chlorophyll/ml in 20 mM Hepes (pH 7.5)/15 mM NaCl/400 mM sucrose and illuminated for 30 s at 273 K in the presence of 80 $\mu$M DCMU and 50 mM $(NH_4)_2SO_4$. (B) as (A), but with 100 mM $(NH_4)SO_4$ and without DCMU, illuminated at 200 K for 5 min. EPR conditions as in Fig. 1.

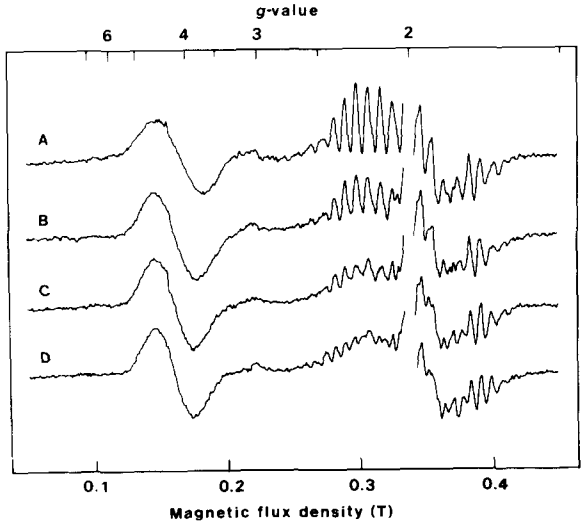



Fig. 3. Modification of the light-induced EPR signals of the $S_2$ state after the reaction of $NH_3$ with the $S_1$ state. The spectra were obtained after pre-equilibration of dark-adapted PS II membranes with (A) 0 mM; (B) 12.5 mM; (C) 50 mM and (D) 150 mM $(NH_4)_2SO_4$. Incubation time before freezing was 1 min. Other conditions as in Fig. 2B.

ing and shift of the $g = 4.1$ signal (Figs. 1C, D and 2).

The effects induced by ammonia were found to be pH-dependent. Considerably higher concentrations (about ten-fold) of $(NH_4)_2SO_4$ were required to modify the EPR signals at pH 6.3 compared to pH 7.5, indicating that ammonia is the binding species for both types of signal modification (not shown).

The effects on the EPR signals described above were specific for ammonia and independent of the type of anion present. Ammonium sulfate and ammonium chloride induced identical changes in the EPR signals, whereas sodium sulfate or sodium chloride were completely ineffective (data not shown).

The interaction of the $S_1$ state with increasing concentrations of $(NH_4)_2SO_4$ resulted in a gradual modification of the EPR signals of the subsequently formed $S_2$ state (Fig.3 ). However, the intensity of the EPR signals determined from their double integrals were constant, which permitted the signals to be resolved into their modified and unmodified components at all stages during the titration. For the multiline signal, this was done by digitally subtracting a weighted unmodified signal from the experimentally obtained spectrum until only a pure modified spectrum remained.

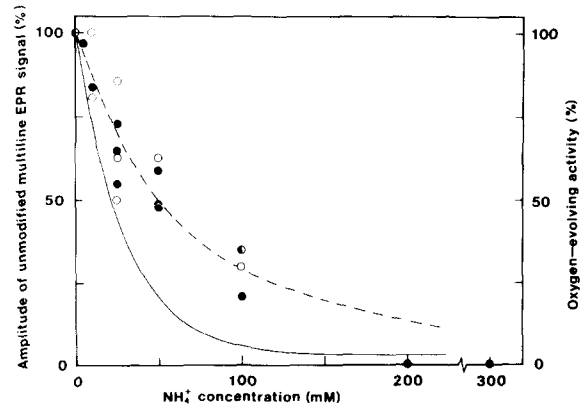



Fig. 4. The modification of the multiline EPR signal after the reaction of $NH_3$ with the $S_1$ state and the effect of chloride. The extent of modification was determined as described in the text. Closed symbols, 15 mM $Cl^-$ present; open symbols, 100 mM $Cl^-$. For comparison, the effect of $NH_3$ on the oxygen-evolving activity is shown. Full line, 15 mM $Cl^-$; broken line, 100 mM $Cl^-$.

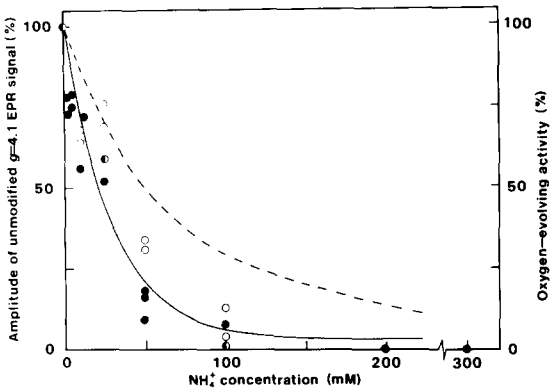



Fig. 5. The modification of the $g = 4.1$ EPR signal after the reaction of $NH_3$ with the $S_1$ state and the effect of chloride. The extent of modification was determined as described in the text. Symbols for EPR signals and oxygen evolution as in Fig. 4.

For the $g = 4.1$ component the degree of modification was determined from the width of the signal. The width of a signal composed of two closely spaced components of different intrinsic widths can be shown to be directly related to the relative amounts of those two components.

Titration curves for the binding of ammonia to the $S_1$ state are shown in Figs. 4 and 5. The concentration of ammonia required to reduce the intensity of the unmodified signal by 50% was different for the two EPR signals. For the multiline signal, 60 mM $NH_4^+$ caused a 50% modification whereas only 20 mM was required for the same modification of the $g = 4.1$ signal.

*The binding of $NH_3$ to the $S_2$ state*

The affinity of the $S_2$ state for ammonia was examined by exposing PS II membranes, given one saturating laser flash, to various concentrations of $(NH_4)_2SO_4$. The same deconvolution technique as described above was used to determine the extent of modification of the multiline signal. Fig. 6 shows that the binding of ammonia to the multiline-modifying site in the $S_2$ state was much stronger than in the $S_1$ state, with an apparent dissociation constant for $NH_4^+$ of about 3 mM.

The S-state dependence of the binding to the site responsible for the modification of the $g = 4.1$ signal was also investigated. For this purpose the $S_2$ state was generated by 200 K illumination of dark-adapted PS II membranes treated with $(NH_4)_2SO_4$. The samples were then thawed in the dark to allow for the reaction of the $S_2$ state with ammonia before being frozen for subsequent EPR analysis. The modification of the $g = 4.1$ signal, under these conditions, was virtually complete at an $NH_4^+$ concentration of 10 mM with an estimated apparent dissociation constant equal to or less than 3 mM (not shown). The modification of the multiline signal using this technique agreed with the results from the flash method described above.

*Comparison between binding and inhibition*

A comparison of the inhibition of the oxygen-evolving activity observed in the presence of ammonia and the binding to the two sites responsible for the modification of the $S_2$-state EPR signals, showed that the binding of ammonia to the multiline signal-modifying site was weaker in the $S_1$ state and stronger in the $S_2$ state than what is expected from the inhibition (Figs. 4, 6). For the $g = 4.1$ signal-modifying site, the apparent dissociation constant in the $S_1$ state had approximately the same value as the $K_i$ for the inhibition of oxygen evolution (20 mM, see Fig. 5) but the binding to this site in the $S_2$ state was much

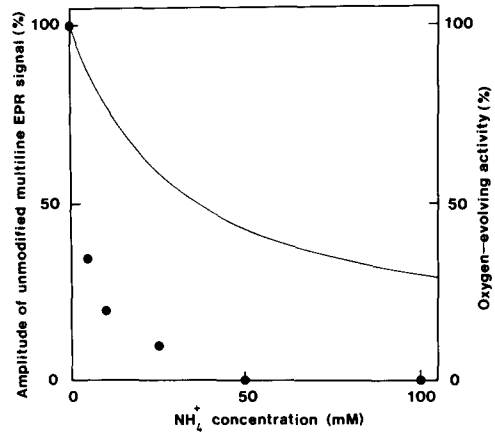



Fig. 6. The modification of the multiline EPR signal after binding of $NH_3$ to the $S_2$ state (closed symbols). The reaction with the $S_2$ state was initiated by exposing PS II membranes to a laser flash followed by incubation of $(NH_4)_2SO_4$ for 15 s, as described in Materials and Methods. $Cl^-$ concentration was 15 mM. Full line, oxygen evolution in the same medium and after the same incubation time with $(NH_4)_2SO_4$.

stronger than the corresponding inhibition (see above). Sulfate, which has been reported to inhibit oxygen evolution weakly, may influence the rates slightly at the highest concentrations of $(NH_4)_2SO_4$ (20% at 200 mM, Ref. 23) but the effect should be much weaker than that of ammonia.

*The effect of $Cl^-$ on the binding of $NH_3$*

The inhibition of oxygen evolution induced by ammonia was decreased in the presence of high concentrations of chloride (Fig. 4). To see if the binding of ammonia to either of the two sites in the $S_1$ or $S_2$ state was affected by chloride in a similar manner, the binding studies were repeated in the presence of 100 mM chloride. No significant effect of chloride on the binding of ammonia to the $S_1$ state could be detected from the extent of modification of either EPR signal (Figs. 4, 5), indicating that the affinity for the anion in this state of the oxygen-evolving complex is much weaker than that of ammonia.

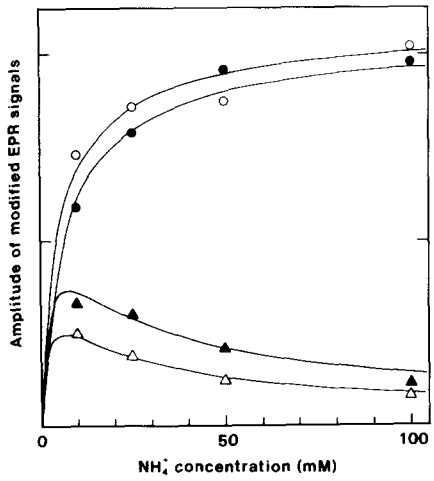



Fig. 7. The amplitudes of the $NH_3$-modified EPR signals after the reaction of $NH_3$ with the $S_2$ state. The reaction was initiated by 200 K illumination, followed by thawing of $(NH_4)_2SO_4$-treated dark-adapted PS II membranes as outlined in Materials and methods. Symbols: circles, modified multiline signal; triangles, modified $g = 4.1$ signal. Closed and open symbols represent the amplitudes at 15 and 100 mM $Cl^-$, respectively. The full lines represent calculated amplitudes of the modified EPR signals using the model in Scheme I and the values for the equilibrium constants given in Table I for the binding of $NH_3$ to the $S_2$ state.

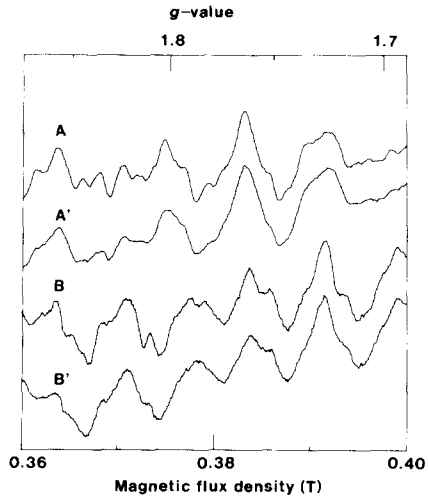



Fig. 8. Effect of $H_2^{17}O$ on the $NH_3$-modified multiline EPR signal from the $S_2$ state. The figure shows part of the signal on the high-field side. The $S_2$ state was produced by illumination at 273 K of dark-adapted PS II membranes, 14 mg chlorophyll/ml in Mes (pH 6.3)/ 15 mM NaCl/400 mM sucrose with 80 $\mu$M DCMU (added dissolved in ethanol in the absence (A), (A') or presence (B), (B') of 300 mM $(NH_4)_2SO_4$. In (A') and (B') the medium contained 42% $H_2^{17}O$. Dark spectra have not been subtracted. Average of 32 scans. Modulation amplitude 0.8 mT, other spectrometer conditions were as in Fig. 1.

When the effect of chloride on the binding of ammonia to the $S_2$ state was examined, the relative amplitudes of the modified multiline and $g = 4$ signals were different at low (15 mM) and high (100 mM) chloride concentrations, although the amplitudes seemed to follow the same general behavior as the concentration of ammonia was increased. The prompt formation of the modified signals at low concentrations of ammonia was followed by a gradual increase of the multiline signal with increasing concentrations, whereas the amplitude of the $g = 4$ signal showed a simultaneous gradual decrease (Fig. 7).

The relative amplitudes of the unmodified EPR signals did not change when PS II membranes were equilibrated in the dark ($S_1$), or after illumination ($S_2$), with chloride at concentrations of 15 or 100 mM in the absence of ammonia.

*The effect of $^{17}O$ on the $NH_3$-modified multiline signal*

We observed earlier that the width of the individual lines of the multiline EPR signal were

slightly broadened in the presence of $^{17}O$-labeled water, which shows that oxygen, possibly as water, is directly coordinated to the manganese [19]. The lines in the ammonia-modified EPR spectrum were also affected by $^{17}O$ (Fig. 8) showing that oxygen is a ligand in the presence of bound ammonia.

## Discussion

Beck and Brudvig, in a recent study [21] of the binding of ammonia to the oxygen-evolving complex, concluded that two sites for ammonia existed, with only one of these accessible in the $S_1$ as well as in the $S_2$ state and which seemed to stabilize the $g = 4.1$ EPR species with bound ammonia (type 2 site). The binding to this site was observed to occur in competition with chloride [24]. The other site, type 1, responsible for the modification of the multiline signal with bound ammonia and possibly identical to the water-splitting, catalytic site, was suggested to be accessible in the $S_2$ state only [18].

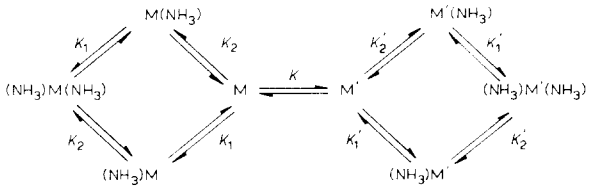
The presence of two sites is confirmed by the data presented in this work, but the overall picture of their accessibility and effect on the $S_2$-EPR signals is significantly different from that observed by Beck and Brudvig. Firstly, the site responsible for the modification of the multiline signal does in fact also bind ammonia in the $S_1$ state, although more weakly, but with virtually identical effects on the $S_2$-EPR signal once this has been formed by low-temperature illumination (Fig. 2). We may, therefore, conclude that the formation of the $S_2$ state does not induce the formation of a new site for ammonia; this is already present in the $S_1$ state with the manganese in a nearly identical environment.

Secondly, we observe another site, one which is related to the $g = 4.1$ EPR signal species. This site is also accessible for ammonia in the $S_1$ and $S_2$ states, with much enhanced affinity in the latter state compared with the other (type 1) site. A binding site with similar properties was also identified by Beck and Brudvig, type 2. There is, however, one important difference between their observation and ours. Beck and Brudvig [21] identified this site from the indirect effect of bound ammonia on the amplitude of the $g = 4.1$ signal, which was otherwise unchanged, whereas we can show that ammonia has a marked influence on both shape and position of the signal when bound. These effects are the same irrespectively of whether the binding takes place in the $S_1$ or $S_2$ states. Thus, this site is also present with similar properties in both oxidation states of the oxygen-evolving complex, with the exception of the value of the binding constant.

A detailed explanation for the discrepancies in results cannot be given at this stage, but it seems clear that a difference in the photosynthetic material used can be ruled out. When the studied the binding of ammonia to the $S_1$ state under conditions similar to those used by Beck and Brudvig and using the same type of medium, we could easily confirm the $NH_3$-induced shift in intensity of the EPR signals and the lack of effect on the multiline signal. Therefore, it is likely that the deviations in observation can be traced to the use of different cryoprotectants (ethylene glycol vs. sucrose). Agents used for this purpose have indeed been found to alter the equilibrium between the two conformational states of the oxygen-evolving complex [28], possibly by affecting the affinity for chloride which is known to influence the relative amplitudes of the EPR signals [24,28]. However, one should not rule out the possibility that cryoprotectants may have other effects as well (see below).

Evidence that the site associated with the $g = 4.1$ EPR signal is common for ammonia and anions, such as chloride also in our case, can be found from the effect of chloride on the binding of ammonia to the $S_2$ state (Fig. 7). In the presence of high concentrations of chloride, the multiline signal is favored at the expense of the modified $g = 4.1$ signal, which implies a competition between chloride and ammonia in the $S_2$ state. In view of these findings and despite the differences in details, it is probably safe to assume that the $g = 4.1$ signal-modifying site is identical to the type 2 site of Beck and Brudvig.

The results presented herein allow a simple model detailing the binding of ligands, particularly $NH_3$, to the oxygen-evolving complex in the $S_1$ and $S_2$ states (Schemes I). The model is an extended version of that put forward by Beck and Brudvig [21] formulated to deal with the new




Scheme I.

observations. The model is general enough to accommodate the results of earlier work.

Firstly, the model, which can be characterized as a general two state – two site model, takes into account the presence of two conformations (M and M′ in Scheme I) of the oxygen-evolving complex, one (M) associated with the multiline in the $S_2$ state, the other (M′) with the $g = 4.1$ signal. These conformations must be present both in the $S_1$ as well as in the $S_2$ state, since they can be observed after treatments which favor the trapping of either state (Fig. 1). The distribution of intensity of the two signals after low-temperature illumination of dark-adapted PS II membranes should reflect the equilibrium between the two conformations in the $S_1$ state. The equilibrium constant was found earlier by double-integration of the two EPR signals [17]. The amplitudes of the signals following freeze-trapping of the $S_2$ state should, in a similar manner, give an indication of the equilibrium situation in this state.

Secondly, two different ligand-binding sites are assumed to be associated with each conformer: the type 1 and type 2 sites, retaining the designations introduced by Beck and Brudvig (the subscripts of the dissociation constants in Scheme I refer to these sites). Chloride, bound to the type 2 site is suggested to favor the conformation (M) associated with the multiline signal (the equilibrium constant $K$ in Scheme I is dependent on the chloride concentration). The lack of effect of chloride at concentrations between 15 and 100 mM on the position of the equilibrium in the $S_1$ and $S_2$ states may indicate that the site is saturated under these conditions. Other anions, such as fluoride and nitrate, which are likely to bind to the same site as chloride [13,24,26] and which have been observed to enhance the formation of the $g = 4.1$ signal, are suggested to bind with higher affinity to the M′ conformation. In addition to the above-mentioned indirect effect on the conformational equilibrium via the anion affinity, cryoprotectants may directly influence the intrinsic equilibrium constant.

Ammonia, bound to the type 1 site in the M conformation (dissociation constants $K_1$ and $K_1'$), induces a modification of the multiline EPR signal. We have earlier demonstrated by the effect of $^{17}O$-labeled water on the multiline EPR signal that oxygen derived from water is directly ligated to the manganese of the oxygen-evolving complex in the $S_2$ state [19]. It is entirely possible that water itself is bound to manganese in this state of the oxygen-evolving complex, since there are indications that exchange of water with the medium can take place even in the $S_3$ state [29]. The observation that $H_2{}^{17}O$ also broadens the $NH_3$-modified multiline signal (Fig. 8) is conclusive evidence that oxygen, possibly as water, is ligated to manganese also when ammonia is bound. This may in turn indicate that ammonia has, at the most, caused a partial displacement of water from the catalytic site. Such a displacement may follow a conformational change induced by the binding of ammonia to a site distinct from the manganese. Recent experiments using $Sr^{2+}$ have provided evidence for such binding coupled to conformational changes affecting the multiline signal [20].

Several observations argue against an anion-binding role for the type 1 site: replacement of chloride by bromide in the $S_1$ state has no effect on the multiline signal [30], and chloride seems unable to reverse the modification of this signal by ammonia bound to this site in the $S_2$ state [25].

Our results show that cryoprotectants appear to have a direct effect on the binding properties of the type 1 site. In an ethylene-glycol-containing medium [18], the affinity for ammonia in the $S_1$ state is lower than in the presence of sucrose, effectively preventing modification of the multiline EPR signal. A comparison with earlier-presented data [21] indicates that ethylene glycol reduces the affinity for ammonia at this site also in the $S_2$ state.

Ammonia is proposed to bind to the type 2 site in both conformers in competition with chloride, inducing a modification of the $g = 4.1$ signal in the $S_2$ state of the M′ conformer. The dissociation constants related to this site ($K_2$ and $K_2'$) should,

TABLE I

EQUILIBRIUM (DISSOCIATION) CONSTANTS REFERRING TO SCHEME I FOR THE BINDING OF $NH_3$ TO THE OXYGEN-EVOLVING COMPLEX IN SPINACH PS-II PARTICLES

The values ($K_1$, $K_1'$) are expressed in terms of concentration of $NH_4^+$. The values for $NH_3$ ($pK_a = 9.3$) are about 50-times lower at the pH used (7.5). Values are in mM.

| | $[Cl^-]$ | $K$ [a] | $K_1$ [b] | $K_2$ [b] | $K_1'$ [b] | $K_2'$ [b] |
|---|---|---|---|---|---|---|
| $S_1$ | 15, 100 | 0.33 | 60 | 20 | 150 | 10 |
| $S_2$ | 15 | 0.33 | 3.3 | 10 | 200 | 1.5 |
| | 100 | 0.33 | 3.3 | 10 | 200 | 2.5 |

[a] From Ref. 17. The equilibrium shows no chloride dependence between 15 and 100 mM $Cl^-$.

[b] Values obtained from fitting Scheme I to the experimental data in Figs. 4–7.

consequently, be dependent on the chloride ion concentration (see below).

The absence of a chloride effect on the modification of the $g = 4.1$ signal in a sucrose-containing medium indicates that the affinity for ammonia in the $S_1$ state is high compared to that of chloride. Also, at this site, the affinity for ammonia is much larger in the $S_2$ state than in the $S_1$ state, which was also noted by Beck and Brudvig [21]. In order to explain the effect of chloride on the equilibrium between the two conformers in the $S_2$ state in the presence of ammonia (Fig. 7), the affinity also for chloride must have been changed radically, compared to that of the $S_1$ state where no such effect was seen. This indicates a general increased affinity for ligands at the type 2 site in going from the $S_1$ to the $S_2$ state. Table I summarizes the apparent dissociation constants for $NH_3$ in the $S_1$ and $S_2$ states calculated using Scheme I to obtain agreement with the experiments. Because, in most cases, chloride is more weakly bound than ammonia, chloride will only have a pronounced effect on the dissociation constant for ammonia in the M′ conformation of the $S_2$ state. One interesting result of these calculations is the indication that the affinity seems to be determined by the oxidation state of the manganese in the $S_2$ state; a binding site with the corresponding manganese center more oxidized compared to the $S_1$ state will bind ammonia much more strongly than a center with the oxidation state the same as in the $S_1$ state, which will have almost unchanged binding properties. This can easily be rationalized in the model presented in Ref. 17, where each signal is associated with a specific metal center.

An S-state-dependent affinity for ligands is also indicated by the deviation between the $K_1$ for the inhibition of oxygen evolution by ammonia (20 mM) and the dissociation constants for ammonia in the $S_1$ and $S_2$ states. Luminescence experiments have suggested that the inhibited step in the oxygen-evolving process is the oxygen-release reaction, i.e., the $S_3$ to $S_0$ transition [31]. If the binding properties of the $S_3$ state determine the inhibition, the results are consistent with a decrease in the affinity for ammonia going from the $S_2$ to the $S_3$ state. From the deviation between inhibition and spectroscopic change, it may be argued that the binding of ammonia to the two sites is unrelated to inhibition. However, the correlation, although only qualitative, between effects on the $g = 4.1$ signal and inhibitory action by agents as different as anions and amines, in addition to the results from chloride competition, strongly suggests that at least the type 2 site is involved in inhibitory action. It is also likely that the structural changes observed in the $S_2$ state, after binding of ammonia to the type 1 site, will affect the activity to some degree if they remain during the catalytic cycle.

It has been suggested that the conformation characterized by the $g = 4.1$ EPR signal in the $S_2$ state represents an inactive form of the oxygen-evolving complex, which is promoted by the presence of inhibitors [24]. Interestingly enough, an increase in the concentration of $NH_3$ is not followed by a general conversion to this conformation in the $S_1$ state, and in the $S_2$ state the amplitude of the modified $g = 4.1$ signal actually decreases while the concentration of the 'multiline conformation' is favored with increasing concentration of $NH_3$ (Fig. 7). This apparently aberrant behavior, however, is a consequence of the presence of two separate binding sites for ammonia on the two conformers and is easily accounted for by the model in Scheme I, with the equilibrium constants given in Table I.

## Acknowledgements

We would like to express our gratitude to Prof. Tore Vänngård for stimulating discussions and valuable criticism. This work was supported by a grant from the Swedish Natural Science Research Council.

## References

1 Babcock, G.T. (1987) in New Comprehensive Biochemistry (Amesz, J., ed.), Vol. 15 Photosynthesis, pp. 125–158, Elsevier, Amsterdam.
2 Dismukes, G.C. (1986) Photochem. Photobiol. 43, 99–115.
3 Brudvig, G.W. (1987) J. Bioenerg. Biomembr. 19, 91–103.
4 Pulles, M.P.J., Van Gorkom, H.J. and Willemsen, J.G. (1976) Biochim. Biophys. Acta 449, 536–540.
5 Dekker, J.P., Van Gorkom, H.J., Wensink, J. and Ouwehand, L. (1984) Biochim. Biophys. Acta 767, 1–9.
6 Dismukes, G.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 274–278.
7 Wydrzynski, T. and Sauer, K. (1980) Biochim. Biophys. Acta 589, 56–70.
8 Yachandra, V.K., Guiles, R.D., McDermott, A., Britt, R.D., Cole, J., Sauer, K. and Klein, M.P. (1987) J. Phys. C8, 1121–1128.
9 Srinivasan, A.N. and Sharp, R.R. (1986) Biochim. Biophys. Acta 850, 211–217.
10 De Groot, A., Plijter, J.J., Evelo, R., Babcock, G.T. and Hoff, A.J. (1986) Biochim. Biophys. Acta 848, 8–15.
11 Hansson, Ö. and Andréasson, L.-E. (1982) Biochim. Biophys. Acta 679, 261–268.
12 Brudvig, G.W., Casey, J.L. and Sauer, K. (1983) Biochim. Biophys. Acta 723, 366–371.
13 Casey, J.L. and Sauer, K. (1984) Biochim. Biophys. Acta 767, 21–28.
14 Zimmermann, J.L. and Rutherford, A.W. (1985) Biochim. Biophys. Acta 767, 160–167.
15 Cole, J., Yachandra, V.K., Guiles, R.D., McDermott, A.E., Britt, R.D., Dexheimer, S.L., Sauer, K. and Klein, M.P. (1987). Biochim. Biophys. Acta 890, 395–398.
16 De Paula, J.C., Beck, W.F. and Brudvig, G.W. (1986) J. Am. Chem. Soc. 108, 4002–4009.
17 Hansson, Ö., Aasa, R. and Vänngård, T. (1987) Biophys. J. 51, 825–832.
18 Beck, W.F., De Paula, J.C. and Brudvig, G.W. (1986) J. Am. Chem. Soc. 108, 4018–4022.
19 Hansson, Ö., Andréasson, L.-E. and Vänngård, T. (1986) FEBS Lett. 195, 151–154.
20 Boussac, A. and Rutherford, A.W. (1988) Biochemistry 27, 3476–3483.
21 Beck, W.F. and Brudvig, G.W. (1986) Biochemistry 25, 6479–6486.
22 Sandusky, P.O. and Yocum, C.F. (1983) FEBS Lett. 162, 339–343.
23 Sandusky, P.O. and Yocum, C.F. (1984) Biochim. Biophys. Acta 766, 603–611.
24 Beck, W.F. and Brudvig, G.W. (1988) Chem. Scr., 28A, 93–98.
25 Andréasson, L.-E. and Hansson, Ö. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. 1, pp. 503–510, Martinus Nijhoff, Dordrecht.
26 Ono, T., Nakayama, H., Gleiter, H., Inoue, Y. and Kawamori, A. (1987) Arch. Biochem. Biophys. 256, 618–624.
27 Franzén, L.-G., Hansson, Ö. and Andréasson, L.-E. (1985) Biochim. Biophys. Acta 808, 171–179.
28 Zimmermann, J.L. and Rutherford, A.W. (1986) Biochemistry 25, 4609–4615.
29 Radmer, R. and Ollinger, O. (1986) FEBS Lett. 195, 285–289.
30 Yachandra, V.K., Guiles, R.D., Sauer, K. and Klein, M.P. (1986) Biochim. Biophys. Acta 850, 333–342.
31 Velthuys, B.R. (1975) Biochim. Biophys. Acta 396, 392–401.