Low-Temperature Interactions of NO with the S_1 and S_2 States of the Water-Oxidizing Complex of Photosystem II. A Novel Mn-Multiline EPR Signal Derived from the S_1 State†

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ABSTRACT: The spin-1/2-carrying NO molecule interacts with both the S_1 and S_2 states of the water oxidizing complex. The intermediates of the interaction can be resolved and trapped by NO treatment at subzero temperatures. At −30 °C and in the presence of approx. 500−700 μM NO, S_1 loses the ability to yield by illumination an EPR active S_2-state with an approximate half-time of 40−60 min. At longer incubation times ($t_{1/2}$ = 4−5 h), an intense new multiline signal develops. The new signal has a hyperfine splitting similar to the S_2 multiline [Dismukes, G. C., & Siderer, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 274−278], but a modified shape with intense lines on the high field side. The NO modified S_1 state can act as a low-temperature electron donor yielding an EPR silent state upon illumination at 200 K. NO interacts also with the S_2 state of the water oxidizing complex rapidly at temperatures as low as −75 °C, to yield an EPR silent state. The rates of the latter interaction show analogies to the ammonia binding to the S_2 state. It is possible, however, that NO, unlike ammonia, destabilizes the S_2 state. On the basis of preliminary experiments with varying chloride concentrations in the range 0.1−50 mM, the S_1 multiline state is attributed to binding of NO at a chloride sensitive site on the Mn cluster. The rapid interactions with the S_2 state as well as the intermediate binding to the S_1 state are less well understood at present, but they are tentatively assigned to the chloride-insensitive site of ammonia binding in the Mn cluster.

The active site of the water oxidizing complex of Photosystem II (PSII)[1] is thought to be a cluster of four manganese atoms. During sequential absorption of photons by PSII the water oxidizing complex undergoes four one-electron oxidation steps, S_0-S_1, .., S_3-S_4, coupled to the release of molecular oxygen [for recent reviews, see Debus (1992), Bricker and Ghanotakis (1996), and Britt, 1996). After a period of dark adaptation the majority of the PSII centers relax to the S_1 state. It is generally agreed that this is an integer spin state. Previous attempts to probe this EPR-silent state have been by parallel mode EPR (Dexheimer & Klein, 1992) and indirectly through the effects on the saturation properties of tyr Y_D• (Koulougliotis et al., 1992). Single-electron oxidation of S_1 produces the half-integer-spin S_2 state, which is EPR active and gives a characteristic multiline signal at $He^{(l)}$ temperatures (Dismukes & Siderer, 1981) and under certain conditions an alternative signal at g = 4.1 (Casey & Sauer, 1984; Zimmermann & Rutherford, 1984). These signals and variants of them [summarized in Zheng and Dismukes (1996)] have been valuable in assaying and understanding basic features of the complex, but there is a strong need for alternative signals that would reduce the uncertainty in the data sets used to simulate this unique cluster.

In earlier studies, it was shown that NO binds reversibly to the PSII acceptor side nonheme Fe^{2+} producing a characteristic EPR signal at g = 4, and also to tyr Y_D• producing an EPR silent species (Petrouleas & Diner, 1990). The latter interaction, which is fast and reversible, is slowly succeeded by the formation of a nitroso−tyrosine complex (Sanakis et al., 1996). This acts as a donor to PSII and can be oxidized by light to an iminoxyl radical. Recently, Szalai and Brudvig (1996) observed that incubation with NO eliminates the ability to trap the so called S_3* EPR signal, attributed now to an interaction between Mn in the S_2 state and tyr Y_Z (Gilchrist et al., 1995; Tang et al., 1996 and references therein). The authors have attributed this effect to an interaction of NO with tyr Y_z•. The earlier studies have also provided preliminary evidence for an interaction of NO with the Mn complex. Incubation of PSII preparations in the S_1 state with NO eliminates the ability to light- induce the S_2 multiline signal (Petrouleas & Diner, 1990). Also, NO at low concentrations (K_m approx. 30 μM in spinach chloroplasts at 25 °C) blocks charge recombination, following a light flash in the presence of DCMU. This effect, which was monitored by fluorescence studies, was attributed to the donor side. NO either acts as a donor, or stabilizes an oxidized donor. As NO is a small neutral molecule with a spin of 1/2, its binding to the Mn cluster would not only offer new spin states accessible through EPR spectroscopy, but would also provide a powerful probe in studies of inhibitor/substrate binding at this site.

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[1] Abbreviations: PSII, Photosystem II; BBY membranes, thylakoid membrane fragments enriched in PSII; S-states: S_0, ..., S_4 oxidation states of the water oxidizing complex; tyr Y_Z, tyr Y_D, the fast and slow tyrosine electron donors of PSII; Q_A, Q_B, the primary, secondary plastoquinone electron acceptors of PSII; EPR, electron paramagnetic resonance; MES, 2-[*N*-morpholineethanesulfonic acid]; chl, chlorophyll.

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We have observed that NO interactions with PSII components can proceed at −30 °C but at a significantly lower rate (Goussias et al., 1995). Under these conditions, the Mn cluster in the S_1 state interacts slowly with NO to produce an intermediate state, which in the hours time scale yields a new multiline EPR signal. The S_2 state also interacts directly with NO; this interaction is rapid and can proceed even at −75 °C.

MATERIALS AND METHODS

PSII-enriched thylakoid membranes were isolated from market spinach by standard procedures (Berthold et al., 1981; Ford & Evans 1983). Samples for EPR measurements were suspended in 0.4 M sucrose, 15 mM NaCl, and 20 mM MES, pH 6.5, at 3−3.5 mg of chl/mL. The NO treatment was carried out anaerobically at 0 °C in EPR tubes by slowly bubbling 4 mL of a mixture of NO and N_2 of a given ratio, typically 2/3 or 1/9 in some experiments, for 1 min (Petrouleas & Diner, 1990). This resulted in reproducible concentrations of NO of 0.5−0.7 or 0.1−0.15 mM, respectively, as measured by the characteristic EPR peak of NO in the $g = 2$ region (Petrouleas & Diner, 1990). In certain control experiments, and in order to eliminate the possibility that the results presented here are due to the interaction of NO with difficult to remove oxygen traces, the buffers and the EPR samples were flushed extensively with N_2 prior to the NO treatment. Illumination of the samples was performed with a 340 W projection lamp filtered through a solution of $CuSO_4$. EPR spectra were recorded using a Bruker ER-200D-SRC spectrometer interfaced to a personal computer and equipped with an Oxford ESR 9 cryostat, an Anritsu MF76A frequency counter, and a Bruker 035M NMR gaussmeter.

RESULTS

Effects of Incubation with NO at −30 °C: an S_1-Derived Multiline Signal. Incubation of PSII membranes in the dark with NO eliminates the ability to induce by light the S_2 Mn multiline signal. The rate of this interaction depends on the temperature of incubation and the concentration of NO. At 0−4 °C and 0.5−0.7 mM NO, the ability to induce the multiline signal is lost in about 5 min of incubation. At about 1/4 the above concentration of NO, the interaction is slower and is completed in approx. 30 min. Longer incubation at the same temperature results in no visible changes to the Mn cluster except on the hours time scale where Mn^{2+} signals are detected in apparent correlation with the formation of a tyr Y_D-nitroso species (Sanakis et al., 1996). The interaction of the acceptor side nonheme Fe^{2+} with NO occurs also on the several min time-scale. Therefore, PSII membranes treated with NO for less than 1 min and then frozen retain the ability to form the normal S_2 multiline and acceptor side $Q_A^-Fe^{2+}$ EPR signals upon illumination at 200 K. Subsequent incubation at −30 °C results in interactions similar to the ones at ≥0 °C, but at a much lower rate. In addition, a unique intermediate of the Mn complex is induced at long incubation times. In the presence of 0.5−0.7 mM NO, the acceptor side $g = 4$ Fe^{2+}-NO signal, which is very small following the 1 min NO treatment, develops slowly at −30 °C in the dark and becomes maximum in less than an hour. At somewhat longer times, the ability to form the S_2 multiline by 200 K illumination is lost with an approximate half-time of 40−60 min. At longer incubation times, a new signal

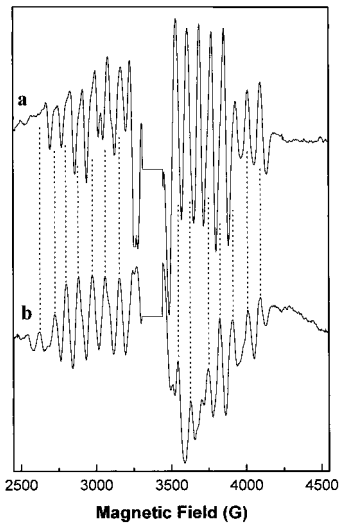


FIGURE 1: Comparison of the S_1 NO induced multiline with the normal S_2 multiline. (a) Sample incubated with NO in the dark at −30 °C for 24 h; the spectrum prior to −30 °C incubation has been subtracted. (b) Untreated sample illuminated at 200 K for 4 min; the spectrum prior to illumination has been subtracted. The central region of the spectra containing contributions from NO or from radicals has been omitted for clarity. EPR conditions: $T = 11$ K; microwave frequency, 9.42 GHz; microwave power, 31 mW; modulation amplitude 25 Gpp.

with hyperfine structure reminiscent of that of the S_2-state Mn multiline gradually develops in the dark, Figure 1a. The new multiline signal evolves with an approximate half-time of ca. 4−5 h. This time is probably overestimated since the free NO concentration, as measured by the characteristic peak in the $g \approx 2$ region of the EPR spectrum (Petrouleas & Diner, 1990), drops during the incubation period. The reason for this is not clear at present. The NO induced multiline signal is compared in Figure 1b with the S_2 multiline signal produced by illumination of an untreated sample at 200 K. Ethanol, 2% v/v, was added to the latter sample in order to reduce formation of the alternative S_2 signal at $g = 4.1$ (Zimmermann & Rutherford, 1986). The two signals have a similar overall width and similar hyperfine splitting (the NO induced signal has a slightly smaller hyperfine splitting). What is distinct in the new signal is the pronounced intense structure in the high-field part of the spectrum. This enhancement in the intensity of the high-field relative to the low-field part of the spectrum is reminiscent (but more pronounced here) of the effect of ammonia on the S_2-state multiline (ammonia additionally reduces the hyperfine splitting) (Beck & Brudvig, 1986; Andreasson et al., 1988; Britt et al., 1989; Boussac et al., 1990). A number of reproducible structural features are also apparent in the low-field peaks of the NO multiline. No $g = 4.1$ signals were observed in any of the NO multiline spectra. The NO-induced multiline appears more intense than the S_2 multiline. This could be due partially to the narrowing of certain lines and partially to inefficiency in the production of the S_2 state in the untreated sample. The new multiline can be also induced with lower concentrations of NO (0.1 mM). It is difficult, however, to estimate the minimum concentrations of NO required since, at low concentrations of NO the kinetics of the multiline formation become slower, and during the long incubation times the free NO concentration decreases drastically.

In addition to the interaction of the NO with the Mn complex, NO interacts with Tyr $Y_D^{\bullet}$ to form initially a weak

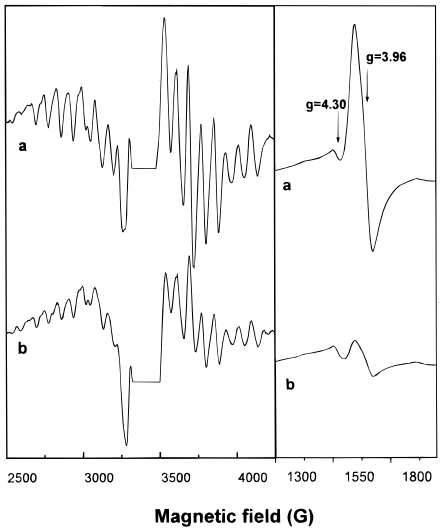


FIGURE 2: Light sensitivity of the NO-induced S_1 multiline (left) and the $g = 4$ Fe^{2+}-NO signal (right) during charge separation at 200 K. Spectra were recorded after 4 h of incubation with NO at −30 °C (a) before and (b) after illumination at 200 K. The spectrum prior to −30 °C incubation was subtracted. The lower sensitivity of the S_1 signal (donor) relative to the Fe^{2+}-NO signal (acceptor) is due to competitive electron donation by other donors (see text). EPR conditions as in Figure 1.

complex (Petrouleas & Diner, 1990) and at long incubation times the recently reported nitroso species, which upon light induced oxidation yields an iminoxyl radical (Sanakis et al., 1996). The initial step of the binding of NO to tyr Y_D• is fast and occurs during the 1 min treatment with NO at 0 °C. The conversion to the nitroso species, which at 1 °C occurs with a half-time of 3.0−3.5 h (Sanakis et al., 1996), at −30 °C develops with an approximate half-time of 20 h. It is interesting that, in contrast to the room temperature incubation, evolution of the tyr-nitroso species at −30 °C is not accompanied by the release of Mn. Actually, the Mn complex is stabilized in the new multiline producing state throughout the incubation period at −30 °C, which in some experiments exceeded the 48 h.

S_1-NO Multiline as a Low-Temperature Electron Donor. The NO-induced multiline signal decreases upon illumination at 200 K. The extent of the decrease depends, however, on the time of incubation with NO. At short incubation times, the multiline species appears to be the sole electron donor, and illumination reduces the signal intensity to zero. At very long incubation times, the tyr Y_D nitroso species has evolved in a large fraction of centers and appears to be the preferential donor. This is probably due to the lower oxidation potential of the nitroso-tyrosine compared to the parent tyrosine (Sanakis et al., 1996). The effects relating to the nitroso-tyrosine at long incubation times will be examined in a future study. During a 4 h incubation period, a significant fraction (approx. 50%) of the multiline develops, Figure 2a (left), and yet the Y_D-NO species (not shown) is only beginning to develop. The multiline signal decreases significantly upon illumination at 200 K, Figure 2b (left). The corresponding decrease in the Fe^{2+}-NO acceptor-side signal at $g = 4$ as a result of the charge separation (Q_A reduction) (Petrouleas & Diner, 1990) is shown in the spectra in the right of Figure 2. The $g = 4$ in Figure 2a (right) represents practically all centers. Upon illumination the $g = 4$ signal decreases by about 85% showing that charge separation is almost complete (the light insensitive fraction is probably due to the opacity of the sample). The corresponding light sensitive fraction of the NO multiline is about 55% while at the same time a small iminoxyl signal (Sanakis et al., 1996) is induced (not shown). The approx. 50% of the centers, which are in a formal S_1-NO state not exhibiting the NO multiline yet, are potential additional donors. These also produce an EPR-silent S_2 state. Following the 200 K illumination, the full size of the initial NO multiline spectrum is recovered within 15 min dark adaptation at −30 °C. This is compatible with the S_2 to S_1 decay under similar conditions in untreated samples.

Treatments That Do or Do Not Affect the New Multiline Signal. Two conformations of the S_1 state have been suggested to exist (Beck et al., 1985); an "active" one, which is paramagnetic and results in an enhanced relaxation rate of tyr Y_D•, and a "resting" one, which is diamagnetic and evolves with an approximate half-time of 3.5 h (Koulougliotis et al., 1992). The active state should in principle be responsible for the parallel mode EPR signal observed by Dexheimer and Klein (1992), but this correlation has not been confirmed. Van Vliet and Rutherford (1996) have considered the possibility that the paramagnetism of the active state is due to molecular oxygen, the product of water oxidation. We have examined whether short or long dark adaptation would make a difference in the NO-induced multiline signal. Three samples were illuminated by continuous light at 0 °C. The first was dark adapted for 30 min at 0 °C, the second and third were dark adapted at 0 °C for 17 h, the third sample was in addition illuminated again at 0 °C and subsequently was dark adapted at 0 °C for 30 min. The three samples, following the respective treatments, were frozen and the saturation properties of tyr Y_D• were measured. The $P_{1/2}$ at 11 K for the first and third sample was found to be 80 μW and for the second 30 μW. These values are compatible with the first and third sample being in the active and the second sample in the resting state (Van Vliet & Rutherford, 1996). The samples were subsequently incubated with NO for 1 min at 0 °C and variable times at −30 °C. All samples developed sizeable S_1 multiline signals comparable to those of nonilluminated samples and with similar time courses. It is possible that NO speeds up the transition to the resting state, or that binding of NO eliminates any likely conformational differences between the active and resting states.

A sample incubated with NO at −30 °C overnight so as to develop the maximum S_1 multiline signal lost the signal after incubation at 0 °C for 1 min. The signal could be fully recovered by a subsequent relatively short (45 min) incubation at −30 °C. This behavior is not understood at present, but it may be due to an unusual property of certain modes of binding of NO. A similar effect has also been observed with certain NO-induced signals, associated with the acceptor side of PSII (Goussias et al., 1995). The loss of the new multiline at 0 °C and higher temperatures explains why the S_1-NO multiline was not observed in any of our previous studies, although extensive ranges of NO concentration and time of incubation at temperatures above 0 °C were tested.

Longer than 1 min (15 min) preincubation at 0 °C with NO did not appear to speed up the NO multiline formation. The size of the signal induced by a subsequent 3 h incubation at −30 °C was less than 50% of the maximum, comparable to what is observed in samples incubated for only 1 min at 0 °C.

Interaction of NO with the S_2 State. As it was mentioned in the beginning, it is possible to populate the unreacted S_2

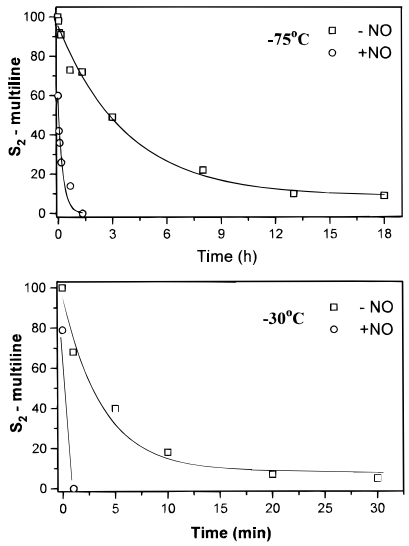


FIGURE 3: Decay of the S_2 multiline signal in the absence and presence of NO at −75 and −30 °C in the dark. The NO treatment was carried at 0 °C for 1 min with a 2/3 (data at −75 °C) or 1/9 (data at −30 °C) NO/N_2 ratio. All samples were illuminated at −75 °C for 4 min prior to studying the decay.

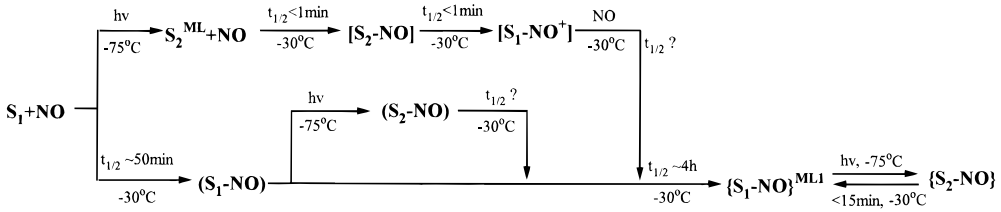
state by illumination, if the initial treatment with NO at 0 °C is restricted to 1 min or less. We examined accordingly the direct effects of NO on this higher state. In order to minimize the S_1−NO interaction and also slow down the S_2−NO interaction, lower NO concentrations (100−150 μM) were used in the studies at 0, −30, and −50 °C. The S_2 state was populated by either 1 min illumination at 0 °C in the presence of atrazine or by 4 min illumination at 200 K in the absence of atrazine. The decay of the multiline was subsequently compared with that in untreated control samples at 0 (samples illuminated at 0 °C) −30, −50, or −75 °C (samples illuminated at 200 K). No multiline could be trapped by the 1 min illumination at 0 °C in the presence of NO, implying that NO interacts with the S_2 state rapidly at this temperature. The decay curves at −30 and −75 °C are presented in Figure 3. At −30 °C, the S_2 to S_1 decay in control samples occurs with an approximate half-time of 3−5 min (or depending on the intactness of the Q_B site longer in some preparations), whereas in the presence of NO the multiline has completely decayed in 1 min. At −50 °C the half-time for the multiline decay is approximately 1.5 min in the presence of NO (data not shown). The decay kinetics are better resolved at −75 °C. The S_2 multiline in the control sample decays with an approximate half-time of 3.5 h while the corresponding half-time in the presence of approx. 0.6 mM NO is 15 min. It is notable that at the elevated NO concentration in this experiment the size of the S_2 multiline following the illumination is about 60% of that of the control. This is probably partially due to an initial S_1−NO interaction during the 1 min treatment at 0 °C and partially to an S_2−NO interaction during the 4 min illumination period. Following the NO induced rapid decay, the S_2 multiline signal can be partially restored, in samples treated with low concentrations of NO, by a second illumination at 200 K (data not shown). At concentrations of NO higher than ca. 0.3 mM, the second illumination does not restore the signal regardless of the length of incubation at −30 °C. On the other hand, no new signals appear during dark adaptation at −30 °C except for the NO-induced multiline which evolves with approximately the same time course as in the nonilluminated samples. A tentative interpretation of the rather complex interaction of the NO with the S_2 state is given in the discussion session.

DISCUSSION

The New Multiline Signal Is Tentatively Attributed to Binding of NO to the S_1 State. The present results show that a new multiline signal is produced slowly during incubation of PSII membranes with NO at −30 °C in the dark. The signal shows similarities to the S_2 multiline, implying that the state producing the species has an oxidation state composition not too different from S_2. It is unlikely, however, that the signal results from the latter state. In dark adapted PSII preparations, the majority of the centers are in the S_1 state. The NO molecule is a weak oxidant (E_m of NO/NO^- 0.25 V vs the NHE; Fontecave & Pierre, 1994) and oxygen, which potentially could form oxidizing intermediates with NO (Wink et al., 1993), was excluded in these studies. Even if such intermediates were formed with difficult to remove oxygen traces, they should be short lived and cannot explain the slow kinetics of the multiline formation. Diffusion of NO is definitely not a rate-limiting factor, as the elimination of the tyr Y_D• EPR signal during the 1 min incubation with NO at 0 °C and the rapid decay of the S_2 multiline at 0 °C indicate that NO reaches all sites prior to freezing. On the other hand, the samples were kept tightly sealed throughout the incubation at −30 °C. Even if air leaked in, O_2 would not diffuse further than a very thin layer at the top of the 2.5 cm-long EPR sample at −30 °C in the hours time scale. The multiline producing state is not likely to be the S_2 for the following additional reasons. Once formed by oxidation of the S_1 state, the S_2 state is rapidly silenced by the free NO. Also, the normal S_2 state is not oxidizable by 200 K illumination, in contrast to the observations in Figure 2. The low-temperature oxidizability of the multiline S_1-NO state and the decay to the initial state in less than 15 min at −30 °C are compatible with the properties of the untreated S_1 state. Although not strictly excluded, it is also unlikely that the state producing the signal is the S_0. The reduction potential of the S_1 state, approx. 700 mV (Vass & Styring, 1991) or less, is much lower than the oxidation potential of NO, 1.3−1.4 V/SCE in aprotic solvents (Fontecave & Pierre, 1994). Even if the reduction of the S_1 state by NO were possible, one would have to explain why this S_0 state is EPR active while the normally produced S_0 state is EPR silent. It would be even more difficult to explain why it takes initially many hours to reduce the S_1 state, while, following the light induced oxidation, the multiline state is recovered within 15 min. On the basis of preliminary evidence, which shows that the new multiline signal is strongly retarded when the NaCl concentration is increased from 15 to 50 mM, we will tentatively assume that the observed slow kinetics is the result of the low probability that NO binds at the Mn site in the S_1 state and replaces perhaps bound chloride (see also Modes of binding of NO). Whether or not internal charge transfer occurs after binding of NO, e.g., S_1-NO → S_0-NO^+, cannot be discriminated at present. Below are simple considerations of the likely spin assignments based on current models of the Mn cluster composition.

If we assume a ($2Mn^{3+}$, $2Mn^{4+}$) composition of the S_1 state (Riggs et al., 1992; Yachandra et al., 1993 and refs therein) with corresponding formal spin assignments [2Mn-($S = 2$), 2Mn ($S = 3/2$)], binding of NO to one of the Mn^{3+} would lead to a spin composition [Mn($S = 2$), Mn-NO($S =$

Scheme 1: Intermediates Generated during the Interaction of NO with the S_1 and S_2 States at Low Temperatures, Following an Initial Brief (∼1 min at 0 °C) Incubation of BBY Samples in the S_1 state with ∼0.6 mM NO[a]



[a] Different kinds of brackets are used to differentiate between potentially different modes/sites of binding of NO. The only EPR detectable states are those labeled ML (multiline signal of the untreated S_2 state) and ML1 (the new multiline signal associated with the S_1 state). Half-times with a question mark are unknown but probably in the several minutes range.

3/2), 2Mn($S = 3/2$)] similar to that of the S_2 state. The difference in the Mn hyperfine contribution of a Mn^{3+}-NO (S_1-NO state) from a Mn^{4+} (S_2 state) may not be significant and within the spectral differences of the two multiline signals in Figure 1. Similar arguments would apply if a ($4Mn^{3+}$) composition of the S_1 state were assumed (Kusunoki et al., 1990; Zheng & Dismukes, 1996). It should be noted, however, that although the total spin of metal−NO complexes can be easily predicted by such simple considerations, the problem of the true oxidation state assignment is not a trivial one [see, e.g., Brown et al. (1995)] and alternative configurations, such as Mn^{n+1}-NO^-, Mn^n−NO, and Mn^{n-1}-NO^+, may have to be considered. It is not clear whether ^{14}N superhyperfine structure accounts for some of the features in the spectra of Figures 1 and 2. Nitrogen hyperfine couplings are, however, not resolvable in any of the cw EPR spectra of the S_2 multiline (Beck et al., 1986; Britt et al., 1989; Andreasson, 1989). Future experiments with ^{15}NO will potentially provide useful information in this respect. At present, it would be also premature to discuss the details of the NO-induced multiline. One of the main features of the spectrum, the higher intensity of the high-field hyperfine lines, could be due to a change in the sign of the Mn hyperfine anisotropy. A similar effect has been observed in the S_2 multiline altered by the binding of NH_3 (Zheng & Dismukes, 1996).

Rapid Interaction with the S_2 State. NO interacts rapidly with the S_2 state to produce an EPR-silent state. At low concentrations of NO, the S_2 multiline signal can be recovered by a second illumination. At concentrations of NO higher than about 0.3 mM, this reversibility is lost. The kinetics and temperature dependence of the NO effect show a striking analogy to the unusual low temperature binding of NH_3 to the same state (Boussac et al., 1990). It is likely, therefore, that the effect of NO to the S_2 state is due to binding to the Mn cluster, possibly at the "S_2-ammonia" site (Beck et al., 1986; Andreasson et al., 1988; Britt et al., 1989; Boussac et al., 1990) or site II in the terminology of Sandusky and Yocum (1984, 1986). Although a direct rapid reduction of the S_2 state by NO seems unfavorable, the bound NO may induce an equilibrium of the type S_2-NO → S_1-NO^+ (e.g., -Mn^{4+}-NO → -Mn^{3+}-NO^+). NO^+ is presumably a labile ligand and at high concentrations of NO is replaced by a neutral NO molecule to form an S_1-NO state, which upon light-induced oxidation produces the EPR-silent states S_2-NO and subsequently S_1-NO^+. The S_1-NO state produced rapidly by the above procedure is presumably the same with the state produced slowly in the dark with a half-time of 40−60 min. In this scheme, the preillumination at 200 K in the presence of NO appears to enhance the reactivity of the S_1 state. This is supported by preliminary NO/NH_3 competition studies. At low concentrations of NO, NO^+ probably dissociates in the S_1 state and is replaced by a physiological ligand. Accordingly, the normal S_2 state is produced by the second illumination. These considerations explain also the earlier observation of the blockage of the charge recombination $S_2...Q_A^-$ following a light flash in the presence of DCMU and low concentrations of NO (Petrouleas & Diner, 1990), as due to the rapid conversion of the S_2 state to S_1 (NO^+). More complicated schemes are, however, possible. The effects of NO to the S_2 state in comparison to the binding of NH_3 will be examined in a separate investigation.

Modes of Binding of NO. The results of the present study can be summarized in Scheme 1. Besides the two main states discussed in the previous paragraphs, a number of intermediates are created during the low-temperature NO treatment and light-induced oxidation. The lower branch in the scheme follows the gradual modifications of the S_1 state by NO. Incubation of BBY membranes with ∼0.6 mM NO for about 1 h (approximate $t_{1/2} \approx 50$ min) at −30 °C (or a few min at 0 °C) results in a state, denoted (S_1-NO), which has lost the ability to form the S_2 multiline by illumination. Charge separation (monitored by the light sensitivity of the Fe^{2+}-NO acceptor side signal) does occur, however, and as no alternative donors can be detected, it is reasonable to assume that this state, by analogy to the longer treated {S_1-NO}^{ML1} state (to be discussed next), advances by illumination at −75 °C to an excited state, (S_2-NO), which is EPR silent. Since the time course of the next step is not affected by this illumination, the (S_2-NO) state must decay in relatively short times (probably on the minutes time scale like the untreated S_2 state) to the ground state (S_1-NO). The (S_1-NO) state on the hours time scale at −30 °C is converted to the {S_1-NO}^{ML1} state, which yields the new multiline signal, denoted ML1. Upon illumination, the {S_1-NO}^{ML1} state advances to the EPR-silent {S_2-NO} state (see the Results for the long term effects on the light sensitivity of the {S_1-NO}^{ML1} state), which decays back to the multiline producing state on the min time scale at −30 °C. The upper branch follows the interaction of the S_2 state with NO. This state, formed by illumination at −75 °C, interacts rapidly with NO (see the Results as well as Figure 3 for details) to form the state [S_2-NO], which presumably converts to the [S_1-NO^+] state. At high concentrations of NO (>0.3 mM), [S_1-NO^+] converts to a state that does not produce the S_2 multiline upon illumination. We will assume that this is the (S_1-NO) state, since the time course for the formation of the {S_1-NO}^{ML1} state is not altered by the prior formation of the [S_2-NO] state in this branch.

There appear to be three main modes of binding of NO, denoted by the three different kinds of brackets in Scheme

1. We will currently assume that a single NO molecule binds to the Mn cluster in each case. The question then is, how many different sites of NO binding exist. There is a large difference in the kinetics and probably in the affinity of the NO binding in the S_1 and S_2 states. Also, the $\{S_2\text{-NO}\}$ state produced by light-induced oxidation of the $\{S_1\text{-NO}\}^{ML1}$ state decays within 15 min to the S_1-multiline state, while the $[S_2\text{-NO}]$ state produced rapidly following the S_2 multiline induction decays to a state having the properties of the EPR-silent (S_1-NO) state. These differences could be assigned to different sites of binding of NO in the two S states. On the basis of the striking similarities in the kinetics and the temperature dependence of binding, it was suggested above that the S_2 site of binding of NO, overlaps with the "S_2-ammonia" site. This site of ammonia binding is chloride insensitive (Andreasson & Hansson, 1987; Sandusky & Yocum, 1984) and the expectation is that the NO binding to the S_2 state will be chloride insensitive, too. This is indeed confirmed by preliminary experiments, which indicate that the rate of binding of NO to the S_2 state at −75 °C is not affected by varying chloride concentrations in the range 0.1−50 mM. The evolution of the $\{S_1\text{-NO}\}^{ML1}$ multiline signal is, however, strongly retarded at 50 mM NaCl. The latter result tentatively supports the assignment of the chloride binding to the Mn cluster [for recent reviews, see Britt (1996), Bricker and Ghanotakis (1996), and Debus (1992)]. It is less easy to understand the (S_1-NO) state. Preliminary investigations with samples incubated with approx. 0.6 mM NO for 1 h at −30 °C and then illuminated at −75 °C indicate that the loss in the S_2 multiline formation (approx. 50%) is not affected by varying chloride concentrations in the range 1−50 mM. This could indicate the existence of a separate chloride-insensitive site. Alternatively, it is possible that the state (S_1-NO) represents binding of NO to the S_2 site, which has a lower affinity or lower accessibility in the S_1 state. This is supported by the observation that, prior formation of the $[S_2\text{-NO}]$ speeds up the conversion to the (S_1-NO) state. The (S_1-NO) state is a precursor to the $\{S_1\text{-NO}\}^{ML1}$ state, which is attributed to the binding of a single NO molecule. We assume, accordingly, that binding of NO to form the (S_1-NO) state is transient and on long incubation the state $\{S_1\text{-NO}\}^{ML1}$ dominates. The Cl^-/NO and NO/NH_3 competitions will be examined more thoroughly in future investigations. Also, although the analogy is remote at present, it will be interesting to examine the possible relation of the (S_1-NO) and $\{S_1\text{-NO}\}^{ML1}$ states with the "one site, two-state model" of Lindberg and Andreasson (1996) for the binding of chloride in Photosystem II. Flash O_2 measurements, although not trivial under an NO atmosphere, are also expected to provide useful insights on the likely overlap of the NO binding sites with the catalytic site.

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