NO Interacts with the Tyrosine Radical $Y_D^•$ of Photosystem II To Form an Iminoxyl Radical†

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ABSTRACT: Incubation of photosystem II, PSII, membranes with NO for a few minutes results in the reversible elimination of the electron paramagnetic resonance (EPR) signal II from the oxidized Tyr $Y_D^•$, presumably due to the formation of a weak Tyr $Y_D^•$−NO complex [Petrouleas, V., & Diner, B. A. (1990) *Biochim. Biophys. Acta 1015*, 131−140]. Illumination of such a sample at ambient or cryogenic temperatures produces no new EPR signals. If, however, the incubation with NO is extended to the hours time range, illumination induces an EPR signal with resolved hyperfine structure in the $g = 2$ region. The signal shows the typical features of an immobilized iminoxyl radical (>C═NO$^•$) with hyperfine values $A_{||} = 44$ G, $A_{\perp} = 22$ G, and $A_{iso} = 29.3$ G. The following observations suggest that the iminoxyl signal is associated with PSII: (a) the signal results from an immobilized species at room temperature probably associated with a membrane-bound component, (b) the abundance of the signal is (sub)-stoichiometric to PSII, (c) the signal is light-induced, (d) some of the treatments that affect PSII (Tris, Ca^{2+} depletion, high-salt wash) severely diminish the size of the signal, and (e) the development of the signal correlates with the release of Mn. In addition, the following observations suggest that the iminoxyl signal results from an interaction of $Y_D^•$ with NO: (a) the evolution of the signal correlates with the loss in reversibility of the Tyr $Y_D^•$−NO interaction and (b) the size of the signal correlates with the initial amount of oxidized Tyr Y_D. It is accordingly proposed that during the incubation with NO, a weak Tyr $Y_D^•$−NO complex is rapidly formed and is then slowly converted to a tyrosine−nitroso adduct. Light-induced oxidation of the latter produces the iminoxyl radical. The nitrosotyrosine is expected to have an oxidation potential significantly lower than the parent tyrosine and can act as an efficient electron donor in PSII even at cryogenic temperatures. It is probably this lowered redox potential of the tyrosine Y_D that explains the release of Mn concomitant with the formation of the nitroso species.

Photosystem II (PSII)[1] contains two redox-active tyrosines, Y_Z and Y_D [for reviews see Babcock et al. (1989) and Hoganson and Babcock (1994)]. Y_Z is the fast intermediate electron carrier between the water-splitting Mn complex and the primary electron donor P_{680}. Tyr Y_D is in a complex, slow redox equilibrium with the Mn cluster [Vass and Styring (1991) and references therein], and in its oxidized form it is stable over a period of minutes to hours. Tyr Y_z has recently been implicated in the water-splitting reactions (H-atom abstractor; Gilchrist et al., 1995; Hoganson et al., 1995). The role of Tyr Y_D remains poorly understood.

In the course of studies of the interaction of NO with PSII components, Petrouleas and Diner (1990) observed that NO under anaerobic conditions interacts reversibly with the oxidized form of Tyr Y_D. Following a brief incubation with NO, the characteristic EPR signal II from $Y_D^•$ disappears. The K_d for this effect was estimated at approximately 3 μM. The Tyr $Y_D^•$ signal could be recovered easily if the NO was removed by evacuation in the dark. Interestingly enough, a similar observation was subsequently made for the interaction of the tyrosine of ribonucleotide reductase with NO (Lepoivre et al., 1992; Roy et al., 1995). Actually, this type of interaction appears to be a general property of phenoxyl radicals (Janzen et al., 1993). Recent pulse radiolysis studies (Eiserich et al., 1995) revealed that the interaction of NO with tyrosine and tryptophan radicals is very rapid with rates of $(1-2) \times 10^9$ M^{-1} s^{-1}, orders of magnitude faster than the known interaction of NO with metal centers.

We report here on a number of observations which show that the reversible rapid interaction of NO with the Tyr $Y_D^•$ of PSII is the first step to a much slower reaction, which is irreversible. This reaction leads to the formation of a Tyr−NO species which can act as an electron donor to PSII. Upon light-induced oxidation of this species, an iminoxyl radical is formed. This is the first example of a chemical modifica-

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[1] Abbreviations: PSII, photosystem II; BBY, thylakoid membrane fragments isolated by a modification of the method of Berthold et al. (1981); MES, 4-morpholineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance; Tyr Y_D and Tyr Y_Z, the slow and fast tyrosines of PSII; Signal II, in this work refers to the EPR signal of the oxidized neutral radical form of Tyr Y_D; P_{680}, a chlorophyll or chlorophyll assembly, which acts as the primary electron donor to PSII; Chl, chlorophyll; free Mn or hexaaquo Mn, Mn^{2+} which shows the characteristic 6-line pattern and is either released to the medium or loosely bound to the reaction center; I.R., iminoxyl radical.

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tion of one of the tyrosines of PSII to produce a photochemically active species.

MATERIALS AND METHODS

PSII Membranes. BBY thylakoid membrane fragments were isolated from market spinach by standard procedures (Berthold et al., 1981; Ford & Evans, 1983). The samples as isolated contain variable fractions of oxidized Tyr Y_D. Full oxidation of Tyr Y_D was accomplished by illumination at 0 °C for 5 min. For the reduction of Tyr Y_D samples at ~0.2 mg of Chl/mL were treated with 10 mM sodium ascorbate for 30 min in the dark at 15 °C. Subsequently, the reductant was washed out and the membranes were concentrated for EPR studies. Typical concentrations of EPR samples were 3−4 mg of Chl/mL in 0.4 M sucrose, 15 mM NaCl, and 50 mM MES, pH 6.5.

Tris Treatment. BBY membranes were diluted to ~0.5 mg of Chl/mL in 1 M Tris, pH 8.1, for 20 min under room light. Tris was then washed out twice and the membranes were suspended in MES buffer, pH 6.5.

NO Treatment. The NO treatment was carried out as described previously (Petrouleas & Diner, 1990). The samples in EPR tubes were bubbled slowly (approximately 3 min) with 3 mL of a gas mixture of NO/N_2 [typically at 2/3 (v/v) ratio unless mentioned otherwise] under anaerobic conditions and subsequently incubated in darkness for various times as indicated. NO was removed by brief (approximately 30 s) evacuation of the EPR tube with a mechanical pump at 0 °C.

Illumination of the samples was carried out with a heat-filtered 340-W project lamp for 4 min at 200 K or for different lengths of time at different temperatures as indicated.

EPR Spectroscopy. EPR spectra at 9.42-GHz microwave frequency were obtained with a Bruker ER-200D-SRC spectrometer equipped with an Oxford ESR 9 cryostat, a Bruker 035M NMR gaussmeter, and an Anritsu MF76A microwave frequency counter and interfaced to a PC.

RESULTS

Observation of a Nitroxide Radical-like EPR Signal. Incubation of PSII samples with NO for a few minutes results in elimination of the EPR signal II from the oxidized Tyr $Y_D^{\bullet}$ due to the formation of an EPR-silent state (Petrouleas & Diner, 1990). Illumination of such a sample at ambient or cryogenic temperatures produces no new EPR signals. If, however, NO is incubated with PSII for a few hours, illumination induces an EPR signal with resolved hyperfine structure in the $g = 2$ region. This signal is shown in Figure 1 after subtraction of the light-insensitive background signals (Goussias et al., 1995). The signal shows the typical features of an immobilized nitroxide radical [see, e.g., Hubbell and McConell (1971) and Freed (1976)]. The requirement of long incubation times with NO is characteristic for the development of the signal. At pH 6.5, the signal develops with approximate half-times of 3.0−3.5 h at 1 °C and less than 2 h at 10 °C.

EPR Parameters of the New Signal Indicate an Iminoxyl Radical. From the spectrum of Figure 1 we obtain the values 44 and 22 G for $A_{||}$ and $A_{\perp}$, respectively, for the principal components of the hyperfine tensor of the ^{14}N nucleus (Hubbell & McConell, 1971). Hence, the $A_{iso} = (A_{||} + 2A_{\perp})/3$ is 29.3 G. This value is much larger than the corresponding value of nitroxide radicals, approximately 15 G, but is typical for radicals of the iminoxyl type (see also Discussion). The spin density on the latter is centered on the nitrogen.

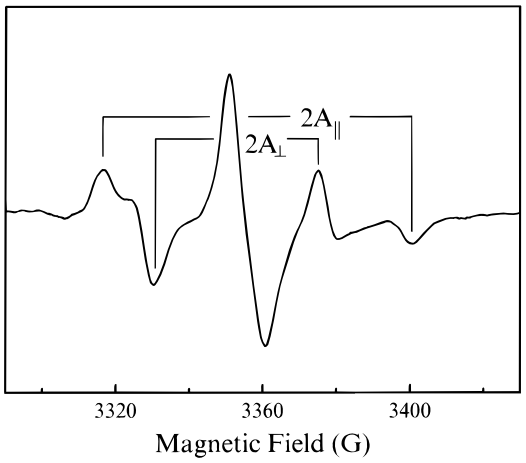

FIGURE 1: Light−dark difference spectrum showing the nitroxide radical-like signal induced in PSII membranes following a 4-h incubation with NO at 10 °C and subsequent illumination at 200 K. EPR conditions: $T = 11$ K; microwave power, 50 μW; modulation amplitude, $4G_{pp}$.

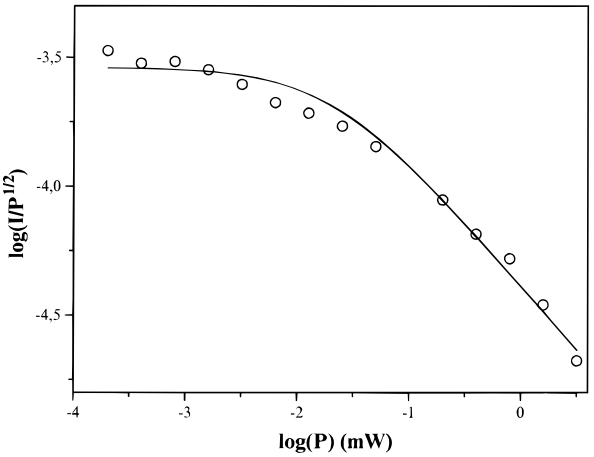

FIGURE 2: Dependence of the iminoxyl radical EPR signal on the microwave power at 11 K. The data were fitted (solid line) with the function $\log (I/P^{1/2}) = C/(1 + P/P_{1/2})^{0.5b}$ with $P_{1/2} = 20$ μW and $b = 1.05$.

The dependence of the signal on microwave power at 11 K is shown in Figure 2. The data are fitted with the function $I = CP^{1/2}/(1 + P/P_{1/2})^{0.5\ b}$, where I is the intensity of the signal, C is a constant which depends on the instrument and the spin concentration, P is the microwave power, $P_{1/2}$ is the power for half-saturation, and b is the "inhomogeneity parameter" (Styring & Rutherford, 1988; Rupp et al., 1978). The $P_{1/2}$ was estimated to be 20 μW at 11 K, while b was found to be 1.05. The value of b suggests a homogeneous line-broadening mechanism. The relatively low $P_{1/2}$ is comparable to the value observed for Tyr $Y_D^{\bullet}$ in Tris-washed samples (Styring & Rutherford, 1988) and suggests that the relaxation properties of this signal are not significantly affected by strong relaxers such as transition-metal centers.

The Iminoxyl Radical Signal Is Associated with PSII. The present preparations are thylakoid membrane fragments enriched in PSII but also containing nonspecific protein/membrane impurities, as well as an estimated contamination

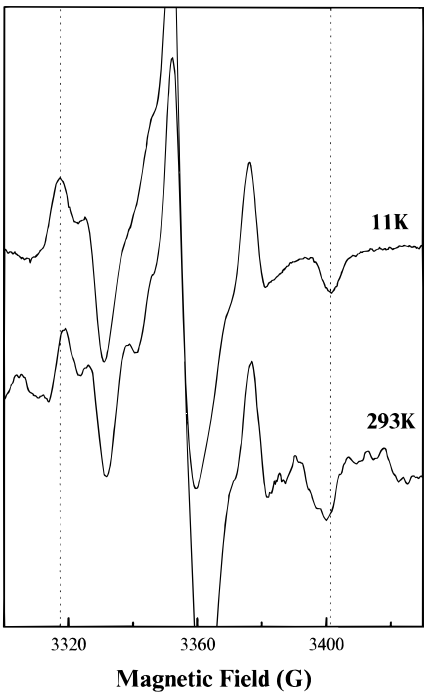

FIGURE 3: Comparison of the iminoxyl radical spectrum at room temperature with the rigid-limit spectrum at 11 K (arbitrarily scaled) indicates that even at room temperature, the spectrum belongs to the slow-motional regime. EPR conditions: microwave power, 8 mW at room temperature and 50 μW at 11 K; modulation amplitude, $4G_{pp}$.

from PSI and the cytochrome b_6/f complex on the order of 10%. It is reasonable, therefore, to ask whether the new signal is associated with PSII.

(a) The signal results from an immobilized species probably associated with a membrane-bound component. This is suggested by comparison of the spectrum at room temperature with the rigid-limit spectrum at 11 K, Figure 3. Apparently, the spectrum at room temperature belongs to the slow-motional regime, and the slight decrease in the separation of the outer hyperfine extrema can be attributed to slow molecular motion at room temperature (Hwang et al., 1975; Freed, 1976; Marsh, 1989). Therefore, in these membrane fragment suspensions the signal must be associated with a membrane-bound component rather than a soluble species.

(b) The abundance of the signal is (sub)stoichiometric to PSII. In all cases examined, the iminoxyl signal appeared substoichiometrically to PSII although the NO concentrations used in some of the experiments were 50-fold higher than the PSII concentration. The comparison was made between the doubly integrated area of the iminoxyl signal and the doubly integrated area of signal II. The latter was maximized by prolonged illumination at room temperature. Under optimum conditions the iminoxyl signal reached an 80% abundance.

(c) The iminoxyl signal is light-induced. This would imply participation in electron transfer. We cannot exclude, however, a direct effect of light, although the fact that the signal can be partially induced even by dim ambient light would favor the first alternative.

(d) Some of the treatments that affect PSII (Tris, Ca^{2+} depletion, high-salt wash) severely diminish the size of the signal. Most notable are the effects of the Tris treatment, which are examined in detail below. These treatments

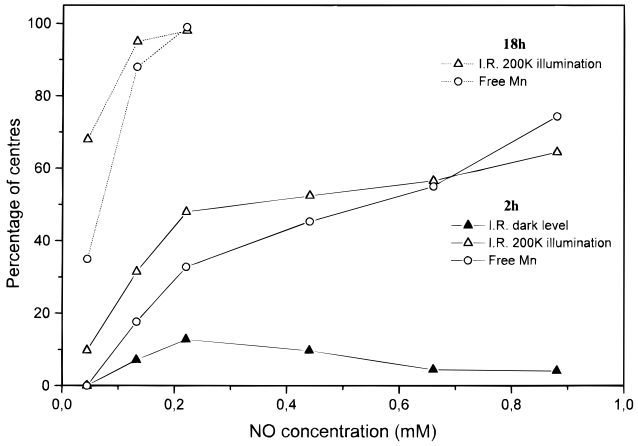

FIGURE 4: Correlation between the development of the iminoxyl radical signal and the appearance of the hexaaquo Mn signal (denoted as free Mn) as a function of the NO concentration at an intermediate time (2-h incubation at 10 °C) and at equilibrium (18-h incubation). Notable is the appearance of an iminoxyl radical signal at low levels prior to illumination.

primarily affect the donor side of PSII. It therefore appears that the iminoxyl signal is associated with the donor side of PSII.

(e) The iminoxyl radical and the Mn complex. As it was noted earlier (Petrouleas & Diner, 1990), incubation with a few hundred micromolar NO eliminates the Mn multiline EPR signal from the S_2 state (Dismukes & Siderer, 1981). This effect occurs much faster than the development of the new signal. The latter appears to correlate with the appearance of a hexaaquo-Mn signal (Miller & Brudvig, 1991), Figure 4, except perhaps at very low concentrations of NO where the free Mn is disproportionately less. Similar correlations were also observed at incubation times of 1 and 4 h, but were not included in Figure 4 for clarity. Also notable in Figure 4 is the presence of a fraction of centers exhibiting the iminoxyl signal prior to illumination. Variable amounts of the dark signal were present in all experiments with intact BBY samples. The signal was absent in Mn-depleted samples by Tris treatment, although illumination induced a sizeable signal as will be discussed in a subsequent section. These observations suggest an interaction between the species giving rise to the iminoxyl radical and the Mn complex.

The Iminoxyl Radical Results from an Interaction of $Y_D^\bullet$ with NO. The location of the iminoxyl species at the donor side of PSII is compatible with the absence of notable influences from the acceptor-side non-heme iron. For comparison, the $P_{1/2}$ of a nitroxide spin-label bound at the Q_B binding site was found to be approximately 200 μW at 15 K (Bocarsly & Brudvig, 1992), a value 10 times higher than the present estimate of 20 μW. The relatively low $P_{1/2}$ observed in the present case suggests that the iminoxyl species is located far from the non-heme iron of the acceptor site. The latter remains intact following the prolonged NO treatment, as shown by the characteristic Fe^{2+}−NO signal at $g = 4$ (Petrouleas & Diner, 1990). It is less easy to examine the distance from the Mn site, as Mn is released.

(a) Evolution of the iminoxyl signal correlates with the loss in reversibility of the Tyr $Y_D^\bullet$−NO interaction. NO has been shown to bind readily at the Tyr $Y_D^\bullet$ site of PSII, eliminating the so-called EPR signal II dark. The interaction with the Tyr $Y_D^\bullet$ is easily reversed and the signal II dark

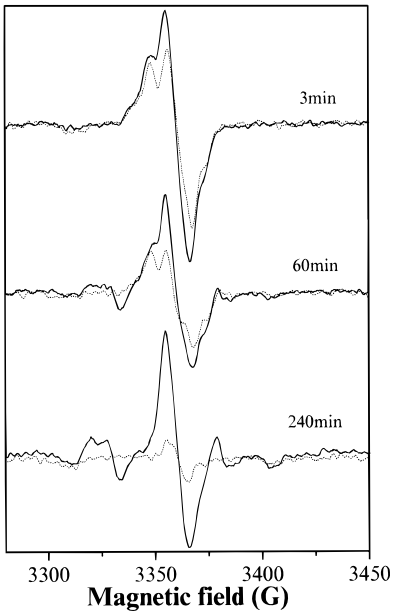

FIGURE 5: Correlation between the loss in reversible binding of NO to Tyr $Y_D^{\bullet}$ and the development of the iminoxyl signal. BBY samples at pH 6.5 were incubated with an NO/N_2 mixture of 2/3 ratio at 10 °C for variable times as indicated. Dotted traces: recovery of signal II following removal of NO by evacuation. Continuous traces: induction of the iminoxyl signal by subsequent illumination of the samples at 200 K. EPR conditions: $T = 11$ K; microwave power, 200 nW; modulation amplitude, $4G_{pp}$.

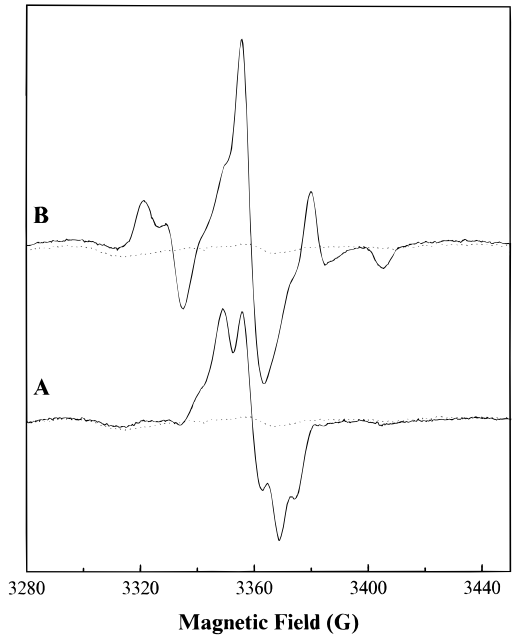

FIGURE 6: Dependence of the iminoxyl signal on the redox state of Tyr Y_D. BBY samples with reduced (approximately 10% of maximum) (A) and maximum (B) signal II were incubated with 2/3 NO/N_2 gas mixture for 4 h at 10 °C. Free NO was subsequently removed by evacuation. EPR spectra were recorded prior to (dotted traces) and after (solid traces) illumination at room temperature. EPR conditions: $T = 11$ K; microwave power, 50 μW; modulation amplitude, $4G_{pp}$.

can be recovered by pumping off NO in darkness (Petrouleas & Diner, 1990). The extent of the reversibility appears, however, to depend on the time of incubation with NO. In Figure 5 we examine the size of signal II recovered following different times of incubation with NO. Three samples with the same initial level of signal II dark were incubated with a gas mixture, NO/N_2 (2/3), at 10 °C for 3 min, 1 h, and 4 h, respectively. Separate experiments (not included) showed that in all cases signal II dark was fully eliminated. NO was subsequently removed by evacuation and the EPR spectra were recorded (Figure 5, dotted trace). Signal II is fully recovered (by comparison with the untreated control, not shown) in the sample that was incubated for a brief time with NO, but it is only partially recovered after a 1-h incubation and is almost eliminated in the sample that was incubated for 4 h with NO. Illumination of the three samples at 200 K (Figure 5, continuous traces) induces the following changes. The sample that was incubated for a brief time with NO behaves similarly to untreated samples in that the Mn multiline signal from the S_2 state is induced at similar intensities (not shown), and in addition, a narrow radical signal at $g = 2$ is added to the signal II spectrum, probably from chlorophyll, acting as an alternative donor in a minority of centers. However, the ability to form the multiline or alternative Mn signals from the S_2 state is lost if incubation with the present concentration of NO is extended beyond 10−15 min. The iminoxyl radical signal is induced in amounts increasing with the time of incubation, as expected on the basis of the results presented above. What is interesting to note here is that there appears to be a correlation between the loss in reversibility of the NO binding to $Y_D^{\bullet}$ and the development of the iminoxyl signal. Actually, the integrated signal intensities of the continuous line traces in Figure 5 are equal if we correct for the small contribution from the chlorophyll cation radical. It is notable, in addition, that loss of the reversibility of signal II is not due to simple chemical reduction of $Y_D^{\bullet}$ (see below). Most likely, NO interacts further with Tyr $Y_D^{\bullet}$ to form a more stable species. It is possible that upon illumination this species converts to the iminoxyl radical. If this is true, then there should be a correlation between the initial amount of oxidized Tyr Y_D and the size of the iminoxyl signal.

(b) The size of the iminoxyl signal correlates with the initial amount of oxidized Tyr Y_D. It was noted earlier, by comparison with the maximum level of signal II, that the iminoxyl signal is substoichiometric to PSII. We have actually observed, by examination of a large number of preparations, that the abundance of the signal never exceeds the initial dark level of signal II. This is exemplified by the experiment shown in Figure 6. Ascorbate-treated BBY membranes before (signal II level approximately 10% of maximum, sample A) and after illumination at room temperature (maximum signal II, sample B) were treated with a 2/3 NO/N_2 gas mixture for 4 h at 10 °C. Free NO was subsequently removed by evacuation. EPR spectra prior to and after illumination at room temperature are shown in Figure 6. No signal II appears in both samples in the dark after removal of free NO. The effects of illumination at room temperature are the following. In the sample with minimum signal II before the NO treatment, a near-maximum signal II is restored while the yield of the iminoxyl signal is small. In the sample with maximum signal II before the NO treatment, a large iminoxyl signal appears in addition to a small contribution from signal II. The latter probably results from incomplete initial oxidation of Tyr Y_D or from a subfraction of centers that underwent reduction of the tyrosine during the NO treatment. Interestingly, the integrated areas of the absorptions (recorded under nonsaturating conditions) in the two samples are approximately the same.

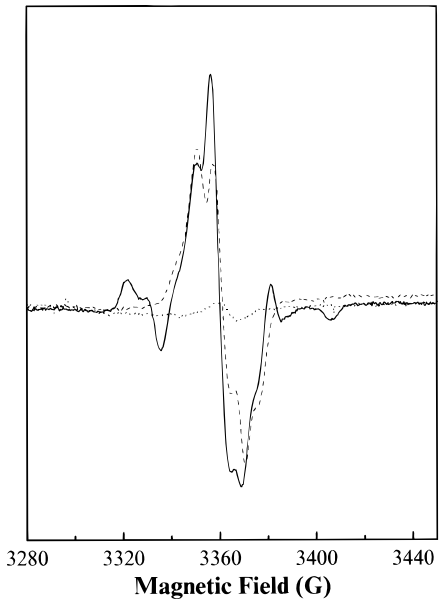

FIGURE 7: Iminoxyl radical yield in Tris-treated BBY. Dashed trace, initial level of signal II maximized by illumination at 0 °C for 5 min. Dotted trace, incubation with NO at 10 °C for 4 h followed by removal of NO. Continuous trace, subsequent illumination at 0 °C for 5 min. EPR conditions: $T = 11$ K; microwave power, 50 μW; modulation amplitude $4G_{pp}$.

(c) Iminoxyl radical signal in Tris-treated samples. It was noted that the iminoxyl signal level is significantly smaller in samples pretreated with Tris or subjected to high-salt washing or calcium depletion. According to the discussion in the previous paragraph, this can be attributed to the observed low levels of signal II dark in these preparations. Indeed, significant amounts of the iminoxyl signal can be induced even in these cases, if the samples are preilluminated so as to maximize signal II dark. This is shown by the following experiment. Tris-treated samples were illuminated for 5 min at 0 °C in order to obtain the maximum $Y_D^\bullet$. $Y_Z^\bullet$, which is also formed under these conditions, was allowed to decay by brief dark adaptation of the samples. Figure 7 (dashed trace) shows the $Y_D^\bullet$ signal. Two separate samples were subsequently treated with 3 mL of a gas mixture of NO/N_2 at 2/3 ratio and stored for 4 h in the dark at 10 °C. Free NO was removed at the end by evacuation, and one of the samples was frozen (Figure 7, dotted trace). Notable is the absence of signal II. The other sample was illuminated at 0 °C for 5 min, followed by brief dark adaptation (Figure 6, continuous trace). Part of signal II reappears, but the iminoxyl signal is also induced, apparently at the expense of signal II. Integration of the spectra recorded at 200 nW (not shown) indicates that the sum of signal II and iminoxyl contributions following the NO treatment and the illumination is within 10%, equal to the initial level of signal II. A qualitative comparison of the spectra in Figures 5 and 6 indicates that the conversion of the $Y_D^\bullet$ to the iminoxyl radical in the Tris-treated samples is less efficient but, nevertheless, significant. The main reason for the difference is probably the decreased stability of signal II in Tris-treated preparations.

A second differentiation concerning the iminoxyl signal in the Tris-treated and in the untreated BBY preparations is the saturation behavior of the signal in the two preparations. The ratio of the signal intensities recorded at 200 nW and 50 μW microwave power is 1.3 in untreated BBY and 6.0 in Tris-treated samples. It is also known that in Tris-treated samples signal II saturates more easily than in normal BBY (Styring & Rutherford, 1988). This behavior is attributed to the Mn cluster contribution to the relaxation properties of the $Y_D^\bullet$. In the present case, Mn is released concomitant to the formation of the iminoxyl species. A possible paramagnetic relaxer could be residual manganese or hexaaquo-Mn^{2+}, but more experimentation is required to clarify this matter.

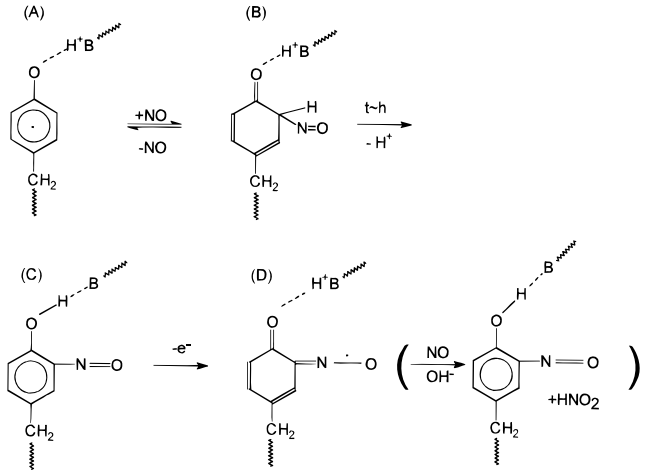

FIGURE 8: Proposed sequence of tyrosyl−NO chemical reactions. Product B is the reversible EPR-silent intermediate. Product C is the slowly forming stable nitroso species, which, upon light-induced oxidation, produces the iminoxyl radical, *D*. The latter can be reversibly reduced by free NO or ascorbate to the precursor nitroso complex.

DISCUSSION

Nature of the $Y_D^\bullet$−NO Interaction. The present results provide evidence for an unprecedented interaction of a tyrosyl radical with NO. This is a slow interaction of NO with Tyr $Y_D^\bullet$ which follows the rapid reversible interaction of the two radicals. Unlike the rapid intermediate, which is EPR-silent, the end product of the slow reaction can be identified in its oxidized state by examination of the hyperfine parameters of the EPR spectrum. The magnitude of the A_{iso} in particular, 29.3 G, is much larger than the values observed for nitroxide radicals of the type $>NO^\bullet$ (aminoxyl), approximately 15 G, but falls in the range of the values observed for radicals of the type $>C{=}NO^\bullet$ (iminoxyl), 28−31G, (Berndt & Neugebauer, 1987). The difference in the A_{iso} value for the two types of radicals is attributed to the difference in the spin density on the ^{14}N (Bethoux et al., 1964). An interesting analogy can be found with the enzymatic oxidation of nitrosonaphthols (Fischer & Mason, 1986). The one-electron oxidation of the 2-nitroso-1-naphthol produces an iminoxyl radical with parameters very similar to the present case. An even closer structural analogy can be found in the series of 1,2-benzoquinone monohydroxyimine free radicals produced by photolysis of *o*-nitrophenols or by chemical oxidation of nitrosophenols (Green et al., 1975). The 5-methyl-2-oxo-1,2-dihydrobenzene-1-iminoxyl derivative, in particular, represents an accurate chemical model of the present iminoxyl radical. Based on these analogies, we suggest in Figure 8 the likely sequence of chemical reactions between the tyrosyl radical and NO. In species A we emphasize the neutral character of the tyrosyl radical and the H-bonding to a nearby base (Babcock et al., 1989; Rodriguez et al.,

1987), most probably H-189 of the D_2 polypeptide (Tang et al., 1993; Tommos et al., 1993). We suggest that product B is formed initially, corresponding to the rapid reversible interaction of NO with the tyrosyl. The more stable 2-nitrosotyrosyl species C is formed at a much slower rate. Roy et al. (1995) and Eiserich et al. (1995) have suggested species C as one of the products of the reversible interaction of NO with the tyrosyl radical, the other product being the O-nitroso species. While the latter could indeed be a reversible product (it was not included in Figure 8 for simplicity), we feel that it is unlikely that complex C is the rapidly forming reversible species. No oxidation product of the rapid intermediate, assigned to species B by us, could be observed in the present case, while, on the basis of the analogy with the nitrosonaphthols and nitrosophenols oxidation of C should produce an iminoxyl radical. Furthermore, the cleavage of the C−H bond required for product C formation should be a slow process. An additional rate-limiting factor in the present case could be the availability of the neutral base for H-bond formation. Diffusion of this proton out of the site may be a slow process since the tyrosine appears to be well isolated from the aqueous phase, and exchange of this proton with the solvent is a very slow process (Rodriguez et al., 1987). It is likely of course that the latter limitation does not apply in other cases, and the time scale of product C formation may be significantly shorter. It would be interesting to examine below a number of observations in the context of these assignments.

Nitrosotyrosine as a Donor to PSII. The iminoxyl signal can be induced by illumination at 77 or 200 K or at room temperature. The possibility that the production of the iminoxyl species is due to the direct effect of light is considered very unlikely. It would take short-UV irradiation to provide the energy for the nitroso-to-iminoxyl conversion, while the signal in the present case can be induced by ambient light of low intensity. The fact that electron transfer is occurring in PSII under these conditions is shown by the light-induced disappearance of the Fe^{2+}−NO EPR signal at $g = 4$ (Petrouleas & Diner, 1990) or by the induction of the $g = 1.84$ signal (Vermaas & Rutherford, 1984) in samples where the NO was removed prior to the illumination and formate was subsequently added (data not shown). Most likely the nitroso adduct is oxidized by the light-driven reactions in PSII, presumably via P_{680}^+. The iminoxyl-/nitrosotyrosine couple is expected to have a lower redox potential than the parent tyrosyl/tyrosine couple. The nitrosotyrosine is therefore a more efficient electron donor compared to its precursor, Tyr Y_D. This could explain the efficiency of the iminoxyl radical formation at cryogenic temperatures. It should be noted, however, that under certain conditions untreated Tyr Y_D can possibly act as a low-temperature donor, too (Nugent et al., 1987). Consistent with its location at the Tyr Y_D site and also with the lowered redox potential of the nitroso complex is the long-term stability of the iminoxyl radical. Once induced by light, the iminoxyl signal is stable at room temperature over a period of hours, provided that free NO is removed prior to illumination. In the presence of NO, the signal decays rapidly at room temperature, presumably due to reduction by free NO. After its decay, the signal can be reinduced by illumination.

Interaction with the Mn Cluster. NO at concentrations of a few hundred micromolar eliminates the Mn multiline signal from the S_2 state of the oxygen-evolving complex (Petrouleas & Diner, 1990). This effect occurs much faster than the development of the iminoxyl signal. The development of the latter appears to correlate with the release of Mn (present data). The Mn complex is, however, susceptible to prolonged chemical treatments. We have examined how much of the "free Mn" signal could be due to a direct effect of the NO treatment. We have observed that in samples with the Tyr Y_D fully reduced so as not to react with NO, the "free Mn" signal produced after a 4-h incubation with NO was at most 50% of the signal observed in samples with the tyrosine fully oxidized. This level is probably an overestimation of the active centers that were affected by NO, since it may include contributions from inactive or nonspecific Mn. Preliminary investigations indicate that the NO treatment leads to the release of variable amounts of the extrinsic polypeptides, which may account for the Mn release. Therefore, at least 50% of the "free Mn" in Figure 4 resulted from an interaction with the Tyr Y_D−NO species. The fact that Mn does not participate in the chemistry that leads to the formation of the iminoxyl radical is shown by the ability to form this species in the Mn-depleted, Tris-treated samples. The following interesting possibility arises then, which also explains the observed levels of the iminoxyl radical in the dark (Figure 4). It has been suggested that a possible role of Tyr $Y_D^{\bullet}$ is to stabilize the Mn complex by oxidizing the lower oxidation states to the S_1 state (Styring & Rutherford, 1987). Accordingly, the redox potential of Tyr Y_D, 720−760 mV (Vass & Styring, 1991; Boussac & Etienne, 1984), is intermediate to those of the S_0 to S_1 and the S_1 to S_2 transitions. It was noted above that conversion to the nitroso species is expected to lower significantly the redox potential of the tyrosine. Presumably then, the nitrosotyrosine can reduce the S_1 or even the S_0 to lower, less stable S states. During the incubation with NO, as soon as the nitroso complex is formed, it is oxidized by the Mn complex to the iminoxyl species, which in turn is rereduced by the free NO. Incomplete reduction by NO could explain the appearance of iminoxyl signal levels in the dark only in Mn-intact preparations. The oxidation/reduction cycle is probably repeated until all Mn is reduced to the Mn^{2+} state and released to the medium. The release of Mn must be facilitated by likely effects of NO on the peripheral proteins. It is possible, however, that the effects of NO on the peripheral proteins require higher concentrations of NO than the binding to the tyrosine. This could be the reason for the partial protection of the Mn release at low concentrations of NO in Figure 4.

Conclusions and Future Investigations. The present study offers a chemical means for a selective modification of Tyr Y_D. The NO treatment produces a species chemically related to the parent tyrosine, redox-active, with a distinct EPR spectrum in the oxidized state. The application of advanced EPR techniques on this spin probe can offer complementary information to earlier investigations. The loss of the Mn is not necessarily a disadvantage for these studies. It is possible, however, that the intactness of the Mn cluster can be improved in future investigations. The present results offer experimental support to the earlier suggestions concerning the stabilizing role of this tyrosine via a redox equilibrium with the Mn cluster. An obvious extension of this work is also the investigation of the interaction of Tyr Y_Z with NO. Preliminary evidence supporting such an interaction was provided during the present studies of the Tris-treated

samples (data not shown). NO eliminated the ability to trap Tyr $Y_Z^{\bullet}$ by illumination at approximately 240 K (Kodera et al., 1992), while the signal would reappear after removal of NO and illumination. It is possible that NO, which is gaseous at this temperature, can bind rapidly to this tyrosine once the latter is oxidized. Similar results were obtained recently by Szalai and Brudvig (1997), who also observed a quenching of the S3 EPR signal in acetate-inhibited PSII. Investigations of the possibility of an iminoxyl radical formation with this tyrosine are currently in progress. Finally, as the biological interactions of NO with free radicals are currently attracting particular attention, the present results may prove useful to the study of NO−radical interactions in other biological enzymes.

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