Resolution of the Paradox of Ammonia and Hydroxylamine as Substrate Analogues for the Water-Oxidation Reaction Catalyzed by Photosystem II

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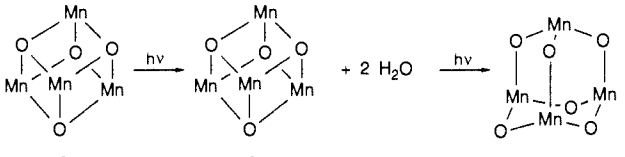
Abstract: The O_2-evolving center of photosystem II (PSII) contains a Mn tetramer complex that serves as the catalyst for the H_2O-oxidation reaction. In a ligand-substitution reaction thought to be analogous to the substrate-binding reaction, NH_3 coordinates to the Mn complex only after formation of the S_2 state, which then exhibits an altered S_2-state multiline electron paramagnetic resonance (EPR) signal. Taking into account this result, Brudvig and Crabtree proposed a model for the mechanism of photosynthetic O_2 evolution, which suggests that coordination of ligands to the Mn complex is governed by the relative electron deficiency of the Mn complex and the Lewis basicity of the attacking ligand. However, the interaction of hydroxylamine with the O_2-evolving center in the S_1 state is not consistent with this mechanism. When spinach PSII membranes are incubated in darkness in the presence of hydroxylamine or *N*-methyl-substituted analogues, the yield of the S_2-state multiline EPR signal obtained after illumination at 210 K steadily decreases. The reaction rate is first-order with respect to the hydroxylamine concentration, depends inversely on the Cl^- concentration, and increases when the pH is raised. These results indicate that the free-base form of hydroxylamine binds to a Cl^--binding site prior to reacting with the Mn complex; subsequently, hydroxylamine probably reduces the Mn complex from the S_1 state to the S_{-1} state through an outer-sphere electron-transfer mechanism. The rate of hydroxylamine's reaction with the Mn complex is reduced about 17-fold with each added *N*-methyl substituent, showing that the binding site is sterically selective for small ligands. NH_3 also binds to the O_2-evolving center in the S_1 state in a manner inversely dependent on the Cl^- concentration, causing the Mn complex to exhibit a $g = 4.1$ EPR signal in lieu of the multiline EPR signal in the S_2 state. The results suggest that primary amines and hydroxylamines bind to the O_2-evolving center in the S_1 state at the same Cl^--binding site. Thus, two ligand-binding sites exist in the O_2-evolving center. One site is located on the Mn complex and is restricted to binding NH_3 and presumably H_2O. This site is assigned to the substrate-binding site. A second site is identified as the Cl^--binding site; primary amines and hydroxylamines compete with Cl^- for coordination to this site. In view of the finding that hydroxylamines bind to the Cl^- binding site, we conclude that hydroxylamines should not be considered as substrate analogues for the H_2O-oxidation reaction catalyzed by PSII.

The O_2-evolving center of photosystem II (PSII) contains four Mn ions[1] that have been proposed to be arranged in a tetrameric, exchange-coupled configuration.[2] The O_2-evolving center catalyzes the four-electron oxidation of H_2O to O_2 by advancing through five intermediate oxidation states S_i, $i = 0$–4.[3] The results of electron paramagnetic resonance[4] (EPR) and X-ray absorption edge[5] experiments demonstrate that the Mn complex functions to accumulate the oxidizing equivalents required for the H_2O-oxidation reaction.[6]

It is also apparent from several EPR experiments that the Mn complex functions as the substrate-binding site of the O_2-evolving center. The Mn complex exhibits a multiline EPR signal in the S_2 state when trapped at low temperature following illumination.[2,4,7-11] When $H_2{}^{17}O$ is present, a small broadening of the hyperfine lines occurs,[7] showing that exchangeable H_2O molecules are directly coordinated to the Mn complex. Further, NH_3, an inhibitor of photosynthetic O_2 evolution,[12] binds to the Mn complex after the formation of the S_2 state, as revealed by a reduction in the hyperfine line spacing of the S_2-state multiline EPR signal.[8,9] It has been proposed that the coordination of NH_3 to the Mn complex in the S_2 state is analogous to the substrate-binding reaction in the H_2O-oxidation cycle.[13]

Scheme I. Model of Brudvig and Crabtree for Structures of Mn Complex Associated with S-State Transitions in PSII (For Clarity, Protein-Derived Ligands Not Shown)



The structural model of Brudvig and Crabtree[14] for the Mn complex of the O_2-evolving center provides a basis for a discussion of the interaction of H_2O analogues with the Mn complex. The Mn complex is postulated to consist of a coordinatively saturated Mn_4O_4 cubanelike structure in the S_0 through S_2 states. In analogy with the finding that NH_3 binds to the Mn complex only after formation of the S_2 state, Brudvig and Crabtree suggested that the binding of substrate H_2O is triggered by the formation of an especially electron-deficient cubane form of the S_3 state. The

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nucleophilic addition of two H_2O molecules in the S_3 state would then yield an adamantane-like Mn_4O_6 structure as shown in Scheme I.[14] The analogous coordination of NH_3 to the Mn complex in the S_2 state, made possible by the greater basicity of NH_3 compared to H_2O, has been proposed to occur via the formation of a μ-imido bridge.[13] The finding that $H_2{}^{17}O$ broadens the hyperfine lines of the S_2-state multiline EPR signal observed in the presence of NH_3[15] can be readily explained by this model because exchangeable μ-oxo ligands are likely to be present simultaneously with the coordinated NH_3 molecule(s). The inhibition of O_2 evolution by NH_3 can be understood, then, if the coordination of *substrate* H_2O at the Mn complex cannot occur when NH_3 is bound. Because NH_3 apparently remains bound to the Mn complex in the S_3 and S_4 states, substrate H_2O molecules cannot be oxidized upon formation of the S_4 state.[16]

The interaction of hydroxylamine with the O_2-evolving center seems to involve a different mechanism. At low concentrations, hydroxylamine has a negligible effect on the rate of O_2 evolution observed under continuous, saturating illumination.[17] Instead, hydroxylamine apparently can react with the O_2-evolving center in the dark-stable S_1 state, causing a two-flash delay in both the O_2-yield[18] and the proton-release[19] patterns detected after a series of saturating light flashes. Several proposals have been advanced to explain the interaction of hydroxylamine with the O_2-evolving center.[17-20] Although it has been suggested that hydroxylamine binds reversibly to the O_2-evolving center in the S_1 state,[19] removal of hydroxylamine from treated O_2-evolving preparations does not eliminate the two-flash delay in the flash-induced O_2-yield pattern.[18a]

On the basis of an extensive series of mass spectrometric measurements with spinach thylakoid membranes, Radmer and Ollinger[20] have proposed that hydroxylamine and hydrazine (which behaves similarly) coordinate to the Mn complex as substrate H_2O analogues in the S_1 state. Their proposal involves a bidentate coordination of hydroxylamine to the substrate binding site in the S_1 state which is then reduced to the S_0 state in the dark. The binding and oxidation of another hydroxylamine molecule by the Mn complex during the first flash of light is proposed to result in the loss of another oxidizing equivalent, leaving the O_2-evolving center 2 equiv behind in the H_2O-oxidation cycle.[20] However, it is not at all clear how hydroxylamine could be expected to coordinate to the Mn complex in the S_1 state while NH_3, a stronger base and a smaller molecule, can bind to the Mn complex only after the S_2 state is formed under conditions of facile ligand exchange.[8,9] This difference in reactivity between primary amines and hydroxylamines has been described as a paradox by Babcock.[1]

If hydroxylamine, however, is capable of binding to the Mn complex in the S_1 state, as proposed by Radmer and Ollinger,[20] then the Brudvig and Crabtree model provides an incomplete picture of the coordination chemistry of the Mn complex. Thus, we focus our attention in this paper on the effects of the binding of NH_3 and hydroxylamine to the O_2-evolving center in the S_1 state in order to further understand how the Mn complex interacts with exogenous ligands. In previous work, we demonstrated that ligand-substitution reactions involving NH_3 occur at two spectroscopically distinct sites in the O_2-evolving center, referred to as the type 1 and type 2 binding sites. The coordination site on the Mn complex, to which NH_3 binds in the S_2 state, was called the type 1 site; this site was assigned to the substrate-binding site of the O_2-evolving center. The binding of NH_3 in either the S_1 or S_2 states to the type 2 site in the O_2-evolving center, but not necessarily on the Mn complex, was detected by observing the production of a stable form of the S_2-state $g = 4.1$ EPR signal with a concomitant reduction in the yield of the S_2-state multiline EPR signal.[9]

In this paper, we have determined the factors that influence the rate of reaction of hydroxylamine with the O_2-evolving center in the S_1 state and those that influence the binding of primary amines to the type 2 binding site in the S_1 state. We resolve the apparently inconsistent behaviors of hydroxylamine and NH_3 by showing that hydroxylamine does not bind to the substrate-binding site in the S_1 state. Rather, hydroxylamine binds to a Cl^--binding site in the O_2-evolving center and, thereafter, probably reduces the Mn complex by two electrons from the S_1 state to the S_{-1} state. Thus, the binding of NH_3 to the Mn complex in the S_2 state and the reduction of the Mn complex in the S_1 state by hydroxylamine are distinct behaviors. The results indicate that hydroxylamine does not behave as a substrate analogue for the H_2O-oxidation reaction catalyzed by PSII.

Experimental Section

Materials. Hydroxylamine hydrochloride was used as received from Eastman Kodak. *N*-Methyl-, *N*,*N*-dimethyl-, and *O*-methylhydroxylamine hydrochloride were used as received from Aldrich. $(NH_4)_2SO_4$ was obtained from Mallinckrodt. 2,5-Dichloro-*p*-benzoquinone (DCBQ), from Eastman Kodak, was recrystallized twice from 95% ethanol, and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), obtained from Sigma, was recrystallized four times from 95% ethanol before use. DCBQ and DCMU solutions were prepared in 95% ethanol immediately prior to use. 2-(*N*-Morpholino)ethanesulfonic acid (MES) and *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid (HEPES) were used as received from Research Organics.

Methods. O_2-evolving PSII membranes were isolated from market spinach leaves according to a Triton X-100 extraction procedure,[21] modified as previously described.[10] O_2-evolving membranes were stored at 77 K in a pH 6.0 buffer solution containing 20 mM MES–NaOH, 15 mM NaCl, and 30% (v/v) ethylene glycol added as a cryoprotectant. All steps in the isolation procedure were conducted in darkness.

EPR samples of PSII membranes were made from extensively dark-adapted, resting state preparations, as previously described.[8] PSII membranes were equilibrated in a sample buffer solution containing 20 mM HEPES–NaOH, pH 7.5, NaCl to obtain the chosen Cl^- concentration (as specified), and 30% (v/v) ethylene glycol; two resuspension and centrifugation cycles were used. The resulting pellet of PSII membranes was resuspended in the same buffer solution to the concentration desired for EPR experiments. As required, NH_3 (in the form of NH_4Cl or $(NH_4)_2SO_4$) or any of the hydroxylamines (as hydrochlorides) were added to the PSII membrane suspension as solutions in the same sample buffer solution. NH_3-treated PSII membrane samples were frozen in liquid N_2, following incubation on ice for 10 min after treatment. Hydroxylamine-treated EPR samples were incubated on ice for the indicated period before being frozen in liquid N_2. All of the steps used in preparing the EPR samples were conducted in darkness.

Sample illuminations were carried out as previously described.[8] EPR spectra were obtained at 8 K with the instrumentation employed previously.[10] All spectra shown are difference spectra (light–dark) obtained through computer subtraction of the dark background spectrum from the postillumination spectrum obtained under the same measurement conditions.

Results

Binding of NH_3 to the O_2-Evolving Center in the S_1 State. The S_2 state can be generated in high yield in untreated PSII membranes by continuous illumination at 210 K and monitored by low-temperature EPR. However, the coordination sphere of the Mn complex, as trapped at low temperatures following illumination at 210 K, remains as it was in the S_1 state.[8,9] Coordination of NH_3 to the Mn complex in the S_2 state requires higher temperatures. We were able to separately observe the effects of the electron-transfer reaction causing oxidation of the Mn complex to the S_2 state and the subsequent coordination of NH_3 to the Mn complex by first illuminating NH_3-treated PSII membranes at 210 K and then warming the sample to 0 °C in darkness, recording an EPR spectrum at 8 K after each step.[9]

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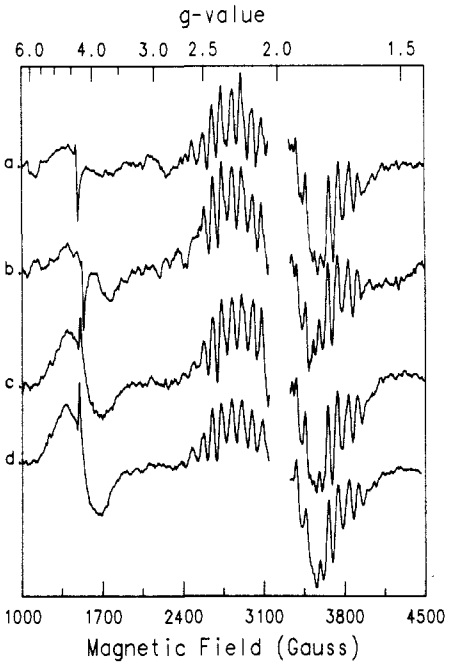

Figure 1. Comparison of S_2-state EPR signals produced in spinach PSII membranes at pH 7.5 by illumination at 210 K for 120 s, showing the effects of Cl^-- and NH_3-binding to the O_2-evolving center in the S_1 state. The $g = 2.0$ region of the spectrum is not shown, owing to interference from EPR signal II_s. EPR spectrometer conditions: microwave frequency, 9.0 GHz; microwave power, 200 μW; field modulation frequency, 100 kHz; field modulation amplitude, 20 G; temperature 8.0 K. Each spectrum is the average of two scans. PSII membranes (5 mg of chlorophyll (Chl)/mL) were treated with 100 μM DCMU: (a) untreated PSII membranes (total $[Cl^-] = 15$ mM); (b) PSII membranes treated with 10 mM NH_4Cl (total $[Cl^-] = 25$ mM); (c) PSII membranes treated with 5 mM $(NH_4)_2SO_4$ (total $[Cl^-] = 0.2$ mM); (d) PSII membranes treated with 10 mM $(NH_4)_2SO_4$ (total $[Cl^-] = 0.2$ mM).

NH_3 also binds to a second site, referred to as the type 2 binding site, in both the S_1 and S_2 states. The binding of NH_3 to the type 2 binding site in the S_1 state can be detected by a conversion of the S_2-state multiline EPR signal, normally observed after illumination at 210 K, into a stable form of the S_2-state $g = 4.1$ EPR signal.[9] A $g = 4.1$ EPR signal with an identical line shape is observed in untreated PSII membranes under our sample conditions following illumination at 130 K; however, the $g = 4.1$ EPR signal observed in untreated samples decays, forming the multiline EPR signal, when the sample is warmed briefly to 210 K.[11,22,23] It was concluded that the binding of NH_3 at the type 2 binding site induces a conformation of the Mn complex in which the $g = 4.1$ EPR signal is stable at 210 K.[9]

Sandusky and Yocum[12] have shown previously that the inhibition of photosynthetic O_2 evolution activity by primary amines also occurs at two different types of binding sites in the O_2-evolving center. Primary amines compete for one type of binding site with Cl^- while only NH_3 binds to a manner independent of Cl^- to a second type of binding site.[12] The latter binding site can be assigned to a binding site on Mn, because only in the presence of NH_3 can the modified S_2-state multiline EPR signal be observed.[9]

The results of Sandusky and Yocum suggest, then, that the binding of NH_3 at the type 2 binding site of the O_2-evolving center in the S_1 state should be sensitive to the Cl^- concentration. To address this possibility, we examined the effect of Cl^- on the yield of the S_2-state $g = 4.1$ EPR signal produced by illumination of PSII membranes at 210 K in the presence of NH_3.

As reported previously, untreated PSII membranes suspended at pH 7.5 exhibit a normal S_2-state multiline EPR signal after illumination at 210 K (Figure 1a).[8] The addition of 10 mM NH_4Cl to PSII membranes at pH 7.5 has very little effect on the EPR signals observed after illumination at 210 K, as is shown in Figure 1b. If 10 mM NH_4^+ is obtained through the addition of $(NH_4)_2SO_4$ at low (0.2 mM) Cl^- concentrations, however, a different result is obtained (Figure 1c); the yield of the S_2-state $g = 4.1$ EPR signal following illumination at 210 K is increased over that observed in the presence of 25 mM Cl^- (Figure 1b). The $g = 4.1$ EPR signal observed in Figure 1c, with 10 mM NH_4^+ present, is 40% larger than that observed at 0.2 mM Cl^- in the absence of NH_4^+ (data not shown),[24] showing that the binding of NH_3 to the O_2-evolving center has enhanced the stability of the $g = 4.1$ EPR signal. Increasing the NH_4^+ concentration to 20 mM, while keeping the Cl^- concentration at 0.2 mM (Figure 1d), results in a still larger yield of the $g = 4.1$ EPR signal. In all four spectra shown in Figure 1, the yield of the $Fe^{II}Q_A^-$ EPR signal at $g = 1.9$, arising from the reduced primary quinone electron acceptor of PSII,[25] is nearly the same, showing that equivalent yields of the S_2 state were obtained.[11] What has changed in the spectra shown in Figure 1 is the proportion of the S_2 state that exhibits either the $g = 4.1$ or the multiline EPR signals.

The results of Figure 1 confirm that the binding of NH_3 to the O_2-evolving center in the S_1 state occurs in a manner dependent on the Cl^- concentration, as was inferred from the results of Sandusky and Yocum.[12] This finding allows the assignment of the type 2 binding site to the Cl^--binding site of the O_2-evolving center. It is likely, then, that other primary amines can coordinate to the O_2-evolving center in the S_1 state to the type 2 or Cl^--binding site, producing a form of the O_2-evolving center exhibiting the $g = 4.1$ EPR signal in the S_2 state. In our previous experiments, however, we did not observe an enhanced $g = 4.1$ EPR signal in the presence of 100 mM CH_3NH_2·HCl because of the high Cl^- concentration.[9] Subsequently, we found that treatment of PSII membranes with the free base form of CH_3NH_2 at 0.2 mM Cl^- produced a large $g = 4.1$ EPR signal at the expense of the S_2-state multiline EPR signal after illumination at 210 K.[24] However, we failed to detect the binding of the bulky amines tris(hydroxymethyl)aminomethane (Tris) or 2-amino-2-ethyl-1,3-propanediol, even at 0.5 mM Cl^-, to the type 2 binding site in either the S_1 or S_2 states.[9] Thus, the type 2 or Cl^--binding site appears to be sterically selective for small Lewis bases in the S_1 or S_2 states.

Reaction of Hydroxylamines with the O_2-Evolving Center. The two-flash delay in the O_2-yield[18] and proton-release[19] patterns upon treatment of spinach thylakoid membranes with low concentrations (<0.5 mM) of hydroxylamine in the dark is consistent with a two-electron reduction of the Mn complex by hydroxylamine. The hypothetical S_{-1} state thus formed from the S_1 state as a result of hydroxylamine treatment would not be expected to yield the S_2 state following low-temperature illumination. Indeed, Casey and Sauer[22] and Guiles et al.[26] have shown that low concentrations of hydroxylamine inhibit the formation of the S_2-state multiline EPR signal in spinach PSII membranes illuminated at 190 K. Experiments by Andréasson and Hansson[15] have indicated that hydroxylamine can also react with the O_2-evolving center in the S_2 state, producing a reduced yield of the S_2-state multiline signal upon a subsequent charge separation at the PSII reaction center; interpretation of their results, however, was complicated by the competition between the reaction of hydroxylamine with the S_2 state and the normal charge-recombination reaction reducing the S_2 state to the S_1 state. We have focused here on the mechanism of the reaction of hydroxylamine and *N*- and *O*-methyl-substituted hydroxylamines with the O_2-evolving center in the dark-stable S_1 state in order to avoid this complication.

Incubation of PSII membranes in the presence of *N*,*N*-dimethylhydroxylamine in darkness at 0 °C for progressively longer periods prior to illumination at 210 K results in a steadily de-

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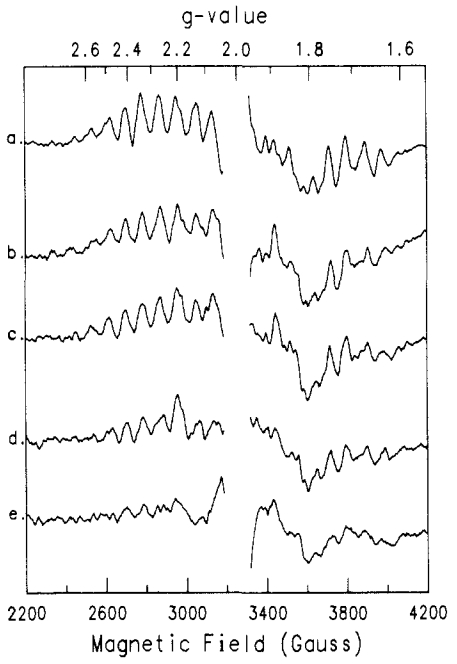


Figure 2. Effect of *N,N*-dimethylhydroxylamine treatment on the yield of the S_2-state multiline EPR signal produced by illumination at 210 K for 120 s. EPR spectrometer conditions were as described in Figure 1. PSII membranes (4 mg of Chl/mL) were treated at pH 6.0 with 100 μM DCMU: (a) untreated PSII membranes (total $[Cl^-]$ = 15 mM); (b)–(e) PSII membranes treated with 18 mM *N,N*-dimethylhydroxylamine (total $[Cl^-]$ = 33 mM), incubated in darkness at 0 °C for (b) 2 min, (c) 5 min, (d) 10 min, and (e) 20 min before freezing in liquid N_2.

creasing yield of the S_2-state multiline EPR signal compared to that observed in the untreated control sample (Figure 2). In each spectrum, however, the yield of the g = 1.9 $Fe^{II}Q_A^-$ EPR signal remains about the same, which shows that the *N,N*-dimethylhydroxylamine treatment does not affect the yield of stable charge separation at the PSII reaction center. We obtained similar results (spectra not shown) when PSII membranes were treated at pH 6.0 with 1.0 mM *N*-methylhydroxylamine or 200 μM hydroxylamine. In each case, the ability to form the S_2-state multiline EPR signal decreased with increasing incubation time with the chosen hydroxylamine species, although different kinetics were observed (vide infra).

It is known that prolonged treatment of O_2-evolving membranes with high concentrations of hydroxylamine causes release of Mn from the O_2-evolving center.[27,28] However, under the less severe conditions we employed, in no case did the loss of the ability to generate the S_2-state multiline EPR signal occur with an associated release of EPR-detectable Mn(II). Furthermore, we have shown that PSII membranes treated with 200 μM hydroxylamine for 5 min at 0 °C produce the S_2-state multiline EPR signal after the *third* illumination at 210 K,[29] showing that the function of the Mn complex is not irreversibly lost as a result of hydroxylamine treatment under these conditions. The decreased yield of the S_2-state multiline EPR signal in the presence of hyroxylamine or *N*-methyl-substituted hydroxylamines cannot, therefore, be attributed to the displacement of functional Mn from the O_2-evolving center. Rather, the decay of the S_2-state multiline EPR signal reflects the kinetics of the reaction of hydroxylamine with the O_2-evolving center during the dark incubation period.

In contrast to the behavior of PSII membranes treated with hydroxylamine, *N*-methylhydroxylamine, or *N,N*-dimethylhydroxylamine, the yield of the S_2-state multiline EPR signal in an *O*-methylhydroxylamine-treated PSII membrane sample (Figure 3b) is nearly identical with that observed in an untreated control sample. Thus, *O*-methylhydroxylamine is incapable of

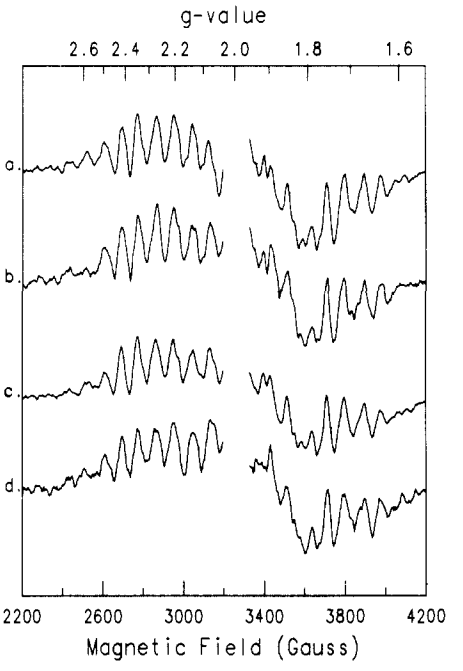
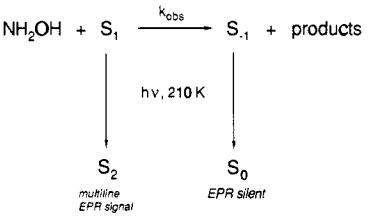


Figure 3. Effect of *O*-methylhydroxylamine treatment (10 min at 0 °C prior to freezing in liquid N_2) on the yield of the S_2-state multiline EPR signal produced in PSII membranes after illumination at 210 K for 120 s or at 0 °C for 30 s. PSII membranes (4 mg of Chl/mL) were treated with 100 μM DCMU at pH 6.0: (a) untreated PSII membranes (total $[Cl^-]$ = 15 mM), illuminated at 210 K; (b) PSII membranes treated with 20 mM *O*-methylhydroxylamine (total $[Cl^-]$ = 35 mM), illuminated at 210 K; (c) untreated PSII membranes (total $[Cl^-]$ = 15 mM), illuminated at 0 °C; (d) PSII membranes treated with 20 mM *O*-methylhydroxylamine (total $[Cl^-]$ = 35 mM), illuminated at 0 °C.

Scheme II. Model for Action of Hydroxylamine and Derivatives on Mn Complex, Resulting in Inhibition of Formation of S_2-State Multiline EPR Signal Usually Formed by Illumination at 210 K in PSII Membrane Samples



reacting in the dark with the O_2-evolving center. This finding is consistent with *O*-methylhydroxylamine's lack of effect on the proton-release pattern, as observed by Förster and Junge.[30] When illumination at 0 °C, rather than at 210 K, is employed to generate the S_2 state, ligand-exchange reactions occur in a facile manner at the Mn complex after the formation of the S_2 state.[8,9] The yields of the S_2-state multiline EPR signal in untreated (Figure 3c) and 20 mM *O*-methylhydroxylamine-treated PSII membranes (Figure 3d) after illumination at 0 °C are not significantly different. Furthermore, the hyperfine line pattern observed in the S_2-state multiline EPR signal formed in the presence of *O*-methylhydroxylamine (Figure 3d) is the same as that observed in untreated samples (Figure 3c). These results argue against the coordination or chemical reaction of *O*-methylhydroxylamine with the Mn complex in either the S_1 or S_2 states.

Kinetics of the Reaction of Hydroxylamine with the O_2-Evolving Center in the S_1 State. The decrease in the yield of the S_2-state multiline EPR signal following illumination at 210 K of PSII membranes treated in darkness with hydroxylamine or *N*-methyl-substituted analogues suggests the model shown in Scheme II. The dark reaction of the hydroxylamine species with the

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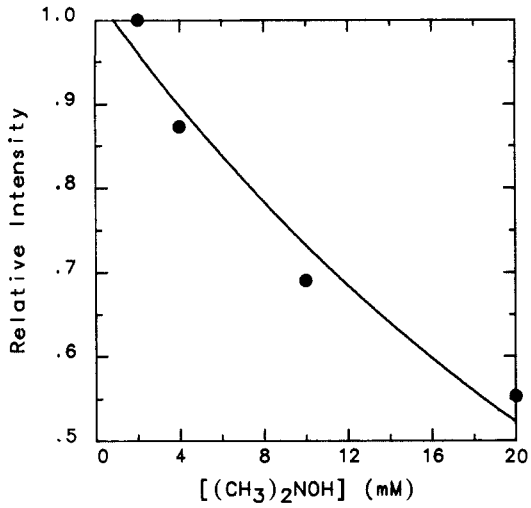

Figure 4. Effect of *N,N*-dimethylhydroxylamine treatment at pH 6.0 (10 min at 0 °C prior to freezing in liquid N_2) on the yield of the S_2-state multiline EPR signal produced in PSII membranes (4 mg Chl/mL) after illumination at 210 K for 120 s. The total [Cl^-] ranged from 17 to 35 mM. The intensity of the S_2-state multiline EPR signal was determined from the sum of the peak-to-peak heights of six hyperfine lines to lower field and four hyperfine lines to higher field of $g = 2$, in the manner employed previously.[8,9] The yield of the multiline signal was normalized relative to that observed with a 2-min incubation.

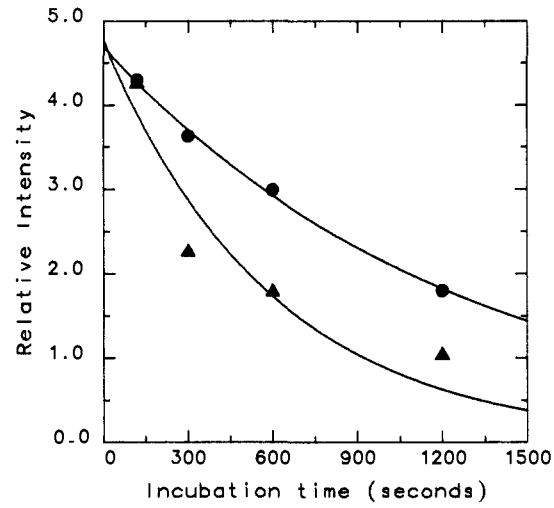

Figure 5. Effect of the length of *N,N*-dimethylhydroxylamine treatment (18 mM, 0 °C) at pH 6.0 (●) and at pH 7.5 (▲) on the yield of the S_2-state multiline EPR signal produced in PSII membranes after illumination at 210 K for 120 s. The intensity of the S_2-state multiline EPR signal was determined as described in Figure 4.

O_2-evolving center is postulated to yield the two-electron-reduced species, S_{-1}, in line with the results of previous proton-release[19] and O_2-yield[18] studies. The S_{-1} state formed by the reaction of hydroxylamine with the O_2-evolving center could be a discrete oxidation state of the Mn complex, as suggested by Saygin and Witt,[31] or the S_{-1} state could represent either the S_0 or S_1 state with bound hydroxylamine molecule(s).[18-20] In another paper, we present results of experiments intended to address the nature of the S_{-1} state;[29] here, we consider only the kinetics of the reaction of hydroxylamine with the O_2-evolving center in the S_1 state.

In Scheme II, the proportion of sites remaining in the unreacted S_1 state decreases with increasing incubation time with hydroxylamine prior to freezing the sample. Thus, illumination at 210 K yields a steadily decreasing amount of the S_2-state multiline EPR signal. The rate equation describing the concentration of unreacted S_1 state as a function of the incubation time in the presence of hydroxylamine, as predicted by Scheme II, is shown in eq 1. If we assume that the concentration of the hydroxylamine

$$-\mathrm{d}[S_1]/\mathrm{d}t = k_{obsd}[S_1][NRR'OH] \quad (1)$$

species NRR'OH is much greater than the concentration of the O_2-evolving centers in the PSII membrane samples, then a pseudo-first-order approximation can be made, yielding eq 2 where

$$-[\ln I_{ml}(t_1) - \ln I_{ml}(t_2)] = k_{obsd}[NRR'OH](t_1 - t_2) \quad (2)$$

$I_{ml}(t)$ is the intensity of the S_2-state multiline EPR signal produced by illumination at 210 K, after a dark incubation in the presence of hydroxylamine for a time t. Equation 2 predicts an exponential decay of the S_2-state multiline EPR signal with incubation time in the presence of hydroxylamine. Further, the rate of reaction should be proportional to the concentration of hydroxylamine; thus, at fixed incubation periods, the intensity of the S_2-state multiline EPR signal should decay exponentially as a function of the concentration of hydroxylamine. The rate constant k_{obsd} obtained by these two methods of analysis should be the same if the reaction obeys the rate law shown in eq 2.

Figure 4 shows the effect of *N,N*-dimethylhydroxylamine treatment for 10 min at 0 °C, in darkness, on the yield of the S_2-state multiline EPR signal. The multiline EPR signal decreases as expected by the model shown in Scheme II in an exponential manner with increasing concentration of the hydroxylamine species. A nonlinear least-squares regression analysis yielded an estimate of $(5.6 \pm 0.9) \times 10^{-2}$ M^{-1} s^{-1} for k_{obsd}, using eq 2 as the regression equation.

For comparison, Figure 5 shows a plot of the intensity of the S_2-state multiline EPR signal as a function of the incubation time in darkness at 0 °C in the presence of 18 mM *N,N*-dimethylhydroxylamine. As predicted by eq 2, the multiline EPR signal decreases exponentially with increasing incubation time. Nonlinear least-squares regression analyses yielded estimates for k_{obsd} of $(4.5 \pm 0.2) \times 10^{-2}$ M^{-1} s^{-1} at pH 6.0 and $(9.6 \pm 3.9) \times 10^{-2}$ M^{-1} s^{-1} at pH 7.5. A comparison of the two values for k_{obsd} obtained for the reaction of *N,N*-dimethylhydroxylamine at pH 6.0, obtained by the dependence of the S_2-state multiline EPR signal on either the concentration of hydroxylamine at a fixed incubation period or the length of incubation with a fixed concentration of hydroxylamine, confirms the validity of the model shown in Scheme II. A similar agreement, within experimental error, for the observed rate constants for the reaction of *N*-methylhydroxylamine (data not shown) was obtained by the two different types of experiments.

Figure 5 shows that an increase in the pH of the suspension buffer solution from pH 6.0 to 7.5 results in more than a doubling of the reaction rate of *N,N*-dimethylhydroxylamine with the O_2-evolving center. This result suggests that the free base amine form of hydroxylamine is the reactive species. An increase in the pH of the suspension medium similarly increases the inhibition of O_2-evolution activity by primary amines.[12]

Since the results of Figure 1 show that the Cl^- concentration extensively limits the extent of binding of NH_3 to the type 2 binding site in the S_1 state, we looked for an effect of Cl^- on the reaction rate of *N,N*-dimethylhydroxylamine with the O_2-evolving center. Figure 6 shows that the reaction rate of *N,N*-dimethylhydroxylamine with the S_1 state is directly proportional to the reciprocal of the Cl^- concentration and extrapolates to zero at an infinite Cl^- concentration. Although the present results do not indicate the mode of inhibition by Cl^- of the hydroxylamine reaction, it seems likely that hydroxylamine binds in lieu of Cl^- at a binding site in the O_2-evolving center prior to reaction. This conclusion is based on the findings of Sandusky and Yocum,[12] who demonstrated the competitive binding of primary amines and Cl^- to a site in the O_2-evolving center.

As discussed above, the binding of primary amines to the Cl^--binding site is apparently sterically restricted, making it difficult to observe by EPR spectroscopy the coordination of bulky primary amines in the S_1 and S_2 states. Table I shows that steric bulk has a similar severe effect on the rate constant for the reaction of hydroxylamines with the S_1 state. *N*-Methyl substitution of

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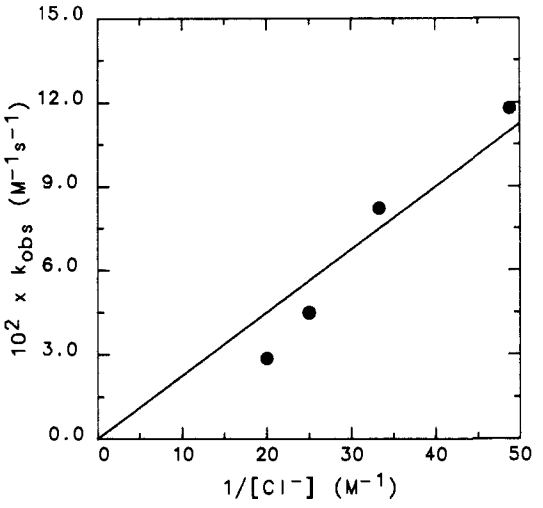

Figure 6. Effect of [Cl⁻] on the observed rate constant for the reaction of *N,N*-dimethylhydroxylamine at pH 6.0 with the Mn complex in the S_1 state. PSII membrane samples were incubated at 0 °C in the presence of 20 mM *N,N*-dimethylhydroxylamine; the total [Cl⁻] was varied through the addition of NaCl. The yield of the S_2-state multiline EPR signal was determined after illumination at 210 K for 120 s, as described in Figure 4. The rate constants were determined by the rate law in eq 2. The solid line corresponds to the best fit, including the origin as a data point.

Table I. Rate Constants for Reaction of Hydroxylamines with the Mn Complex in the O_2-Evolving Center of Photosystem II[a]

compound	k_{obsd}, M^{-1} s^{-1}	factor
NH_2OH	45.6 ± 10.0	1.0
CH_3NHOH	2.95 ± 0.97	1/15.4
$(CH_3)_2NOH$	0.150 ± 0.021	1/304
NH_2OCH_3	no reaction	

[a] Conditions: All rate constants were determined at 0 °C at a Cl⁻ concentration of 15 mM, in the same manner described in Figure 5. The S_2-state multiline EPR signal was produced by illumination at 210 K for 120 s. The rate of reaction for $(CH_3)_2NOH$ was determined by extrapolation from the dependence of k_{obsd} on [Cl⁻].

hydroxylamine decreases the rate constant by about a factor of about 17 for each added methyl group. *O*-Methyl substitution of hydroxylamine yields a complete loss of reactivity, as discussed above, probably owing to electronic, rather than steric, effects. In general, *O*-alkylhydroxylamines are not reactive with respect to oxidations,[32] making it seem very unlikely that they could serve as electron donors to PSII.

Discussion

The experiments discussed in this paper were intended to address the question of the site to which NH_3 and hydroxylamine bind in the dark-stable S_1 state. We have shown that the extent of the binding of NH_3 to the O_2-evolving center and the rate of the reaction of hydroxylamine with the O_2-evolving center both depend inversely on the Cl⁻ concentration. In contrast, the binding of NH_3 to the Mn complex in the S_2 state, producing an altered S_2-state multiline EPR signal, occurs even at high Cl⁻ concentrations.[9,15] Thus, ligand-exchange reactions involving small primary amines and hydroxylamines occur at a site other than the type 1 binding site located on the Mn complex, which has been proposed to be the substrate-binding site of the O_2-evolving center.[9,13]

This finding resolves the paradox posed in the introduction: we conclude that hydroxylamine and its analogues, including probably hydrazine, should not be considered as substrate analogues for the H_2O-oxidation reaction catalyzed by the Mn complex. Moreover, hydroxylamine should be restricted from coordinating to the Mn complex since primary amines larger than NH_3, including CH_3NH_2, do not bind to the Mn complex in the S_2 state.[9] Thus, the detailed topological model of the H_2O-binding site formulated by Radmer and Ollinger,[20] which was founded on the relative reactivity of alkyl-substituted hydroxylamine and hydrazine species with the O_2-evolving center, should not be regarded as pertaining to the substrate-binding site of the O_2-evolving center. It is possible, however, that the model of Radmer and Ollinger[20] applies to the Cl⁻-binding site.

The reaction of hydroxylamine and *N*-methyl-substituted analogues with the O_2-evolving center in the S_1 state can be described with a very simple model. The observed dependence of the reaction rate of *N,N*-dimethylhydroxylamine with the S_1 state on the Cl⁻ concentration suggests than an equilibrium, shown in eq 3, is established prior to formation of the S_{-1} state. The

$$S_1(Cl^-) + NRR'OH \rightleftarrows S_1(NRR'OH) + Cl^- \quad (3)$$

$$S_1(NRR'OH) \rightarrow S_{-1} + \text{products} \quad (4)$$

binding of the hydroxylamine species NRR'OH to the O_2-evolving center displaces Cl⁻ from its site (eq 3); upon binding, the hydroxylamine species is oxidized by the O_2-evolving center through an electron-transfer reaction (eq 4), inhibiting the formation of the S_2-state multiline EPR signal (as in Scheme II). The slower rate, compared to hydroxylamine, at which *N*-methyl- and *N,N*-dimethylhydroxylamine react can be attributed to a steric restriction at the Cl⁻-binding site, which would produce a less favorable equilibrium constant in eq 3.

We have considered the nature of the S_{-1} state formed by the reaction of hydroxylamine with the O_2-evolving center in the S_1 state in more detail elsewhere.[29] The most likely interpretation of our findings is that the S_{-1} state is a distinct oxidation state of the Mn complex. A similar proposal for the nature of the S_{-1} state was made previously by Saygin and Witt.[31] One mechanism for the reduction of the Mn complex to the S_{-1} state would involve the outer-sphere transfer of two electrons from a bound hydroxylamine species at the Cl⁻-binding site to the Mn complex. This reaction apparently does not impair the ability of the Mn complex to undergo further normal S-state advances, since the S_2-state multiline EPR signal can be produced subsequently after three charge separations.[29]

At very high concentrations of hydroxylamine, however, Mn(II) ions are released from the O_2-evolving center, owing to an irreversible disruption of the Mn complex by hydroxylamine. Cheniae and Martin[27] found that the rate of the irreversible inactivation of O_2 evolution in the dark in spinach thylakoid membranes is slowed by *N*-methyl substitution of hydroxylamine; further, they observed that *O*-methylhydroxylamine does not cause inactivation of O_2 evolution in darkness. Cheniae and Martin[27] also concluded that the free base form of hydroxylamine was the inactivating species from a study of the pH dependence of the reaction. Similar results were obtained in PSII membrane samples treated in darkness with hydroxylamines by Tamura and Cheniae,[33] who further demonstrated that the rate of inactivation of O_2 evolution followed first-order kinetics with respect to the hydroxylamine concentration. Kelley and Izawa[28] showed that the ability of hydroxylamine to irreversibly inhibit O_2-evolution depends inversely on the Cl⁻ concentration. Thus, it appears from these results that the irreversible inactivation of O_2 evolution by hydroxylamine at high concentrations also involves a Cl⁻-binding site and the same steric and substitutional factors that control the rate of the reversible dark reduction of the O_2-evolving center to the S_{-1} state by hydroxylamine. However, the results of Tamura and Cheniae[33] show that the rate constant for the inactivation of O_2 evolution by hydroxylamine in darkness is much smaller, by about a favor of 30, than that reported in this paper for the

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reduction of the O_2-evolving center to the S_{-1} state, under similar conditions.

A possible general mechanism for the reaction of hydroxylamine with the O_2-evolving center in dark-adapted PSII membranes, based on the evidence presented in this paper and the results reviewed above, involves at least three steps. The reaction of a hydroxylamine molecule with the O_2-evolving center in the S_1 state would initially produce the S_{-1} species, as described above in eq 3 and 4. Then, a second hydroxylamine molecule would bind at the Cl^--binding site and react at a slower rate with the S_{-1} species, reducing the O_2-evolving center to a yet lower oxidation state. According to the model proposed by Brudvig and Crabtree,[14] the Mn complex is most likely a Mn^{IV}_3-Mn^{III} complex in the S_2 state. The S_{-1} state would then be a Mn^{III}_4 complex. Thus, further reduction of the S_{-1} state by hydroxylamine would necessitate production of a Mn^{II} ion in the Mn tetramer. It is well-known that Mn^{II} complexes are relatively labile, owing to a lack of a ligand field stabilization energy.[34] We propose, then, that the coordination of hydroxylamine at a sterically hindered Cl^--binding site in the O_2-evolving center in the S_1 state precedes the reduction of the Mn complex by hydroxylamine and that the lability of the Mn complex increases as the tetramer is increasingly reduced. This proposal accounts for the inhibition of the Mn-depletion reaction in hydroxylamine-treated thylakoid membranes under moderate intensities of illumination;[35] the S_{-1} state intermediate would be oxidized to a less labile oxidation state while under illumination, thus preventing the loss of functional Mn from the O_2-evolving center.

The binding of primary amines to the O_2-evolving center appears to be influenced by the same factors that control the rate of the reaction of hydroxylamines with the Mn complex. First, the Cl^- concentration affects the ability of NH_3 and CH_3NH_2 to bind at the type 2 binding site. This result suggests that primary amines displace Cl^- from its binding site in a manner like that proposed in eq 3 for the equilibrium involved in the reaction of hydroxylamines with the Mn complex. We have described the competition of amines and Cl^- at the type 2 binding site in detail elsewhere;[24] the binding of NH_3, CH_3NH_2 and F^- in the S_1 state affects the structure of the Mn complex in a manner indistinguishable from the effects of depletion of Cl^- from the O_2-evolving center. Second, similar steric factors appear to influence the binding of primary amines to the type 2 binding site and the rate constant for the hydroxylamine reaction. Our previous failure to detect the binding of bulky primary amines such as Tris to the O_2-evolving center, even at low Cl^- concentrations, is completely consistent with the steric restriction implied by the approximately 17-fold reduction in the rate constant of the hydroxylamine reaction upon *N*-methyl substitution of hydroxylamine. Last, both the primary amines and the hydroxylamines bind to the O_2-evolving center as free-base species, as indicated by the increasing degree of amine inhibition of O_2 evolution[12] and the increasing rate constant for the hydroxylamine reaction with increasing basicity. It is likely, then, that the hydroxylamines and primary amines compete with Cl^- for a common site.

The location of this Cl^--binding site is likely to be quite near to the Mn complex, since an electron is transferred in a facile manner from a bound hydroxylamine species to the Mn complex. This proximity of the Mn complex to the Cl^--binding site could also account for the stabilization by NH_3 and CH_3NH_2 of the form of the Mn complex exhibiting the S_2-state $g = 4.1$ EPR signal; a small, local protein conformational change caused by displacement of Cl^- could alter the exchange interactions in the Mn complex,[2] causing a conversion from the normally observed S_2-state multiline EPR signal to the S_2-state $g = 4.1$ EPR signal. The action of Cl^- as a cofactor of photosynthetic O_2 evolution[28] could well be due to the maintenance of a preferred protein conformation near the Mn complex. Removal of Cl^- from the O_2-evolving center could interfere with the accumulation of oxidizing equivalents, as has been observed in the experiments of Theg et al., Itoh et al., and Ono et al,[36] by perturbing the structure of the Mn complex.[24]

Acknowledgment. This work was supported by the National Institutes of Health (Grant GM32715). G.W.B. is the recipient of a Camille and Henry Dreyfus Teacher-Scholar Award and an Alfred P. Sloan Fellowship. We thank George Cheniae for several helpful comments concerning this work.

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Exciplexes of Ruthenium(II) α-Diimine Complexes with Silver(I)

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Received August 5, 1987

Abstract: Luminescence "quenching" of RuL_3^{2+} (L = 2,2'-bipyridine and 4,7-dimethyl-1,10-phenanthroline) photosensitizers by Ag^+ in aqueous solutions is shown to proceed, not by oxidative electron transfer quenching as previously believed, but predominantly by formation of luminescent exciplexes. Photochemical formation of $Ag^0(aq)$ is <0.02 in water and <0.05 in acetonitrile. Both a normal bimolecular exciplex, $*(RuL_3|Ag)^{3+}$, and a rare termolecular $*(RuL_3|Ag_2)^{4+}$ exciplex are observed. This appears to be the first example of an exciplex formed by a Ru(II) α-diimine sensitizer and of a termolecular metal complex exciplex. Spectral properties of the exciplexes, energetics, and dynamics for these systems are discussed.

Platinum metal complex photosensitizers are invaluable mechanistic tools and promise to function as the photon antenna in photochemical energy conversion or other photocatalytic systems. Much of the work has been devoted to the original $Ru(bpy)_3^{2+}$ (bpy = 2,2'-bipyridine) complex and its analogues. These sensitizers offer considerable flexibility due to the ease of altering their properties by modifying the α-diimine ligand in order to tune excited state energies or redox potentials.[1] A second approach

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