can be described by a vertical reaction coordinate on the left side of Figure 4 that corresponds to a stepwise mechanism.

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**Supplementary Material Available:** Table S1 summarizing rate constants $k_0$ and $k_{cat}$ in water and deuterium oxide (4 pages). Ordering information is given on any current masthead page.

# Turnover Control of Photosystem II: Use of Redox-Active Herbicides To Form the $S_3$ State

**Jeffrey R. Bocarsly† and Gary W. Brudvig***

*Contribution from the Department of Chemistry, Yale University, New Haven, Connecticut 06511.*
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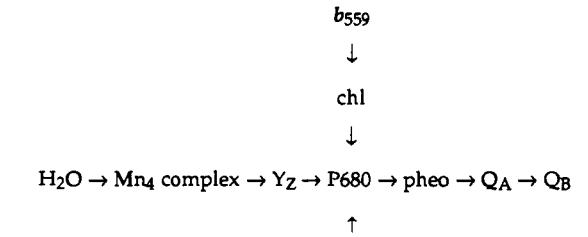
**Abstract:** The $O_2$-evolving center of photosystem II, which contains an active-site tetramanganese–oxo cluster, catalyzes the four-electron oxidation of two water molecules to dioxygen, with the concomitant production of four $H^+$ and four electrons. During catalytic turnover, the manganese–oxo cluster steps through five intermediate oxidation states, which are known as the $S_i$ states ($i$ = 0–4). While methods have been found to manipulate the system into $S_1$ and $S_2$ in high yields, efficient production of the $S_3$ state in good yield at high concentration has not yet been achieved. Previous methods have suffered from the requirement of low protein concentration so that actinic flashes are saturating; the use of temperature to control S-state advancement under continuous illumination, which can lead to S-state scrambling; or the use of herbicides that bind to the $Q_B$ site and restrict the system to one turnover. We describe here a method for the high-yield production of the $S_3$ state in highly-concentrated samples of photosystem II, through the use of electron-accepting herbicides which bind to the $Q_B$ site. Redox-active herbicides can be used, in principle, to limit S-state cycling to any desired number of turnovers, given the appropriate herbicide. This work has fundamental methodological implications not only for the study of photosystem II but also for other multistate redox protein systems.

Photosystem II (PSII) is a multicomponent plant membrane protein complex which utilizes light energy to drive the catalytic oxidation of water.[1] The water-oxidation reaction occurs at an active site containing a tetrameric manganese complex.[2] The manganese complex is the first component in the electron-transfer chain of PSII which consists, in sequence, of a redox-active tyrosine residue ($Y_Z$), the primary chlorophyll electron donor (P680), a pheophytin molecule (pheo), the primary quinone electron acceptor ($Q_A$), and finally a secondary quinone electron acceptor ($Q_B$). A second redox-active tyrosine ($Y_D$), a chlorophyll molecule (chl), and cytochrome $b_{559}$ are alternate electron donors in PSII. Of these alternate donors, cytochrome $b_{559}$ has been shown to donate to $P680^+$ via an intermediary chlorophyll,[3] and $Y_D$ donates directly to $P680^+$.[4] These reactions are summarized in Scheme I.[5]

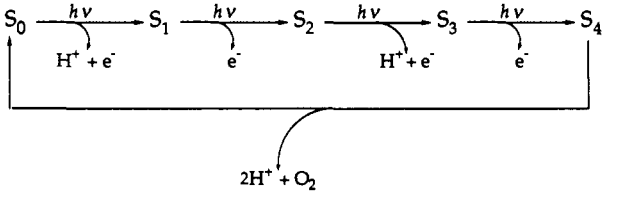
During PSII turnover, the manganese tetramer stores 4 oxidizing equiv produced by the photochemical apparatus of PSII. These oxidizing equivalents are used to oxidize 2 equiv of water to generate four protons and a molecule of dioxygen. Thus, the manganese cluster has five accessible oxidation states through which it cycles during the water oxidation reaction; in the model of Kok,[6] these have been labeled as five S states (Scheme II).

Since the elucidation of the S-state cycle, the accessible states have been the objects of intensive study, both to gain information about the structure of the manganese cluster and to arrive at an understanding of the role of the cluster in the overall chemical and physical scheme of PSII. However, only $S_1$ and $S_2$ are readily accessible from the dark state of the enzyme, which, at physiologic pH and after short dark adaptions, is thought to contain a 3:1 mixture of $S_1$ and $S_0$.[6] After long dark adaptions, $S_1$ is exclusively formed.[7,8] The EPR-active $S_2$ state has been formed by exposure of a dark-adapted PSII sample to single flashes at 273 K[9] or to continuous illumination while freezing[10] or at 195 K.[11] Herbicides such as DCMU that bind to the $Q_B$ site, and thereby restrict PSII to one stable charge separation, have also been used to great

Scheme I




Scheme II




† Present address: Department of Chemistry, University of Connecticut, Storrs, CT 06269.

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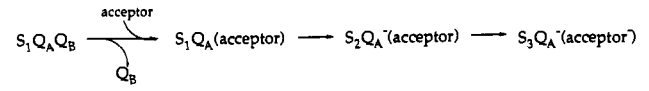
**Scheme III**




advantage to allow continuous illumination to be used to form the $S_2$ state in high yield in highly-concentrated samples. Thus, both the $S_1$ and the $S_2$ states are accessible in good yield in highly-concentrated samples. Study of the $S_0$ and $S_3$ states, however, has been hindered by the difficulty in preparing samples which are both enriched in these states and sufficiently concentrated for spectroscopic study. Previous preparations of these states have been based on either the advancement of samples from the $S_1$ state by two or three actinic flashes[12] to reach $S_3$ or $S_0$, respectively ($S_4$ is an unstable intermediate and rapidly decays to $S_0$), or, in the case of $S_3$, the use of chemical treatments followed by continuous illumination.[13] The former preparations, however, suffer from the requirement that samples have low protein concentration so that the flashes used are saturating, while the latter treatment does not reproducibly produce high concentrations of $S_3$ (estimated $S_3$ yield: either 46% or 66%, depending on method of preparation[13]). This is because the continuous illumination methods use temperature to control S-state advancement, which can lead to scrambling of the states and reduced $S_3$ yield.[11,14] Temperature, in principle, can be used to control S-state advancement, because each stepwise oxidation of the manganese–oxo cluster along with the concomitant reduction of the quinone electron acceptor exhibits a temperature threshold. (The protonation requirements of the reduced quinone may play an important role in setting the temperature threshold for some of the acceptor-side electron-transfer steps.) Temperature control of S-state advancement during continuous illumination, however, is technically difficult to achieve without some S-state advancement beyond the desired state, leading to a significant percentage of scrambled centers in the sample. The present study seeks to address the problem of forming $S_3$ in good yield and high concentration.

Our approach centers on the design and synthesis of molecules which have two features: the ability to accept electrons from the PSII reaction center and the ability to bind to the $Q_B$ binding site with a high binding constant, regardless of oxidation state. Such molecules, when bound in the $Q_B$ site, would allow the $O_2$-evolving center (OEC), prepared in the $S_1$ state, to step through a number of S states equal to the number of accepted electrons, plus an additional step for the one-electron reduction of $Q_A$ (Scheme III). While our focus has been on the production of $S_3$, which would require the bound electron acceptor to participate in a one-electron reaction, in principle PSII could be cycled through any desired number of steps via the use of a multiple-electron-accepting group.

(5) Abbreviations: OEC, oxygen evolving center; chl, chlorophyll; $Y_Z^\bullet$, transient tyrosine radical; $Y_D^\bullet$, stable tyrosine radical; P680, primary electron donor in photosystem II; pheo, pheophytin; $b_{559}$, cytochrome $b_{559}$; $Q_A$, primary quinone electron acceptor; $Q_B$, secondary quinone electron acceptor; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; MES, 2-morpholinoethanesulfonic acid.

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## Experimental Section

**Synthesis.** All reagents and solvents were purchased from commercial sources and were used as received. Grignard reagent was prepared according to standard procedures. NMR spectra of nitroxide-containing products were taken at 250 MHz after reduction of the product using aqueous sodium dithionite. Mass spectra were taken on a Kratos MS-80 mass spectrometer.

**Synthesis of 1,1-Dimethyl-3-phenylurea–TEMPO (1).** 1,1-Dimethyl-3-[4-(chlorosulfonyl)phenyl]urea (0.12 g, 1.6 mmol) and 4-amino-TEMPO (0.15 g, 0.88 mmol) were stirred in $CHCl_3$ (45 mL). Triethylamine (0.25 mL, 1.8 mmol) was added, and the solution was refluxed for 2 h. Triethylamine (0.12 mL, 0.86 mmol) was added again, and the solution was refluxed for an additional 2 h. As concentration in vacuo to ~3-mL volume and storage at −20 °C did not cause precipitation, the solution was evaporated to dryness to completely remove remaining triethylamine. The residue was dissolved in ~2 mL of $CHCl_3$ and was stored at −5 °C, causing precipitation of a light red solid. The solid was filtered from solution, washed with chilled $CHCl_3$, and allowed to air dry (0.034 g, 20% yield). $^1H$ NMR (in $D_2O$, after reduction with $Na_2S_2O_4$): $\delta$ 7.48 (4 H, q, aromatic), 3.38 (1 H, m, methyne), 2.82 (6 H, s, $NCH_3$), 1.60, 1.31 (4 H, dd, methylene), 1.22, 1.16, 1.12, 1.08 (12 H, s, $CH_3$). Mass spectrum (EI): *m/e* (relative intensity) 397 ($M^+$, 1), 367 (3), 352 (55), 338 (100), 322 (49), 310 (2). Anal. Calcd for $C_{18}H_{29}N_4O_4S$: C, 54.39; H, 7.35; N, 14.09. Found: C, 53.87; H, 7.32; N, 13.82.

**Synthesis of (Dimethylureido)phenyl Phenyl Nitroxide (2).** This radical was synthesized by reduction of 3-(4-nitrophenyl)-1,1-dimethylurea with a Grignard reagent.[15] The starting material was synthesized as follows. Dimethylamine hydrochloride (0.50 g, 6.1 mmol) was dissolved in $CHCl_3$ (10 mL). The solution was chilled on an ice bath, and triethylamine (0.93 mL, 6.7 mmol) was added. 4-Nitrophenyl isocyanate (1.0 mL, 6.1 mmol) was slurried in $CHCl_3$ (10 mL), and the slurry was added dropwise to the dimethylamine solution over ~15 min. During this addition a precipitate formed. The solution was stirred for an additional 1.5 h while warming to room temperature. The solid was filtered from solution, washed with $CHCl_3$ and allowed to air dry (0.95 g, 74% yield).

3-(4-Nitrophenyl)-1,1-dimethylurea (0.10 g, 0.48 mmol) was then slurried in THF (10 mL) while equilibrating in a dry ice/acetone bath. A 0.89 M phenylmagnesium bromide solution in THF (4.3 mL, 3.8 mmol) was added rapidly, causing a bright orange solution to form, followed by rapid precipitation. The solution was stirred in the dry ice/acetone bath for 30 min and then was allowed to warm to room temperature over 2 h, during which the precipitate dissolved. The solution was then extracted with saturated aqueous $NH_4Cl$ (40 mL). The layers were separated, and the aqueous layer was extracted three additional times with diethyl ether (3 × 10 mL). The organic layers were combined and concentrated in vacuo to a thick oil. The oil was dissolved in diethyl ether (10 mL), and this solution was dried over $CaSO_4$. The desiccant was filtered from solution, and the solution was again cooled in a dry ice/acetone bath. Silver dioxide (0.23 g, 1.6 mmol) was added, and the solution was stirred while being concentrated in a dry $N_2$ stream for 0.5 h. The solution was then filtered, concentrated in vacuo to ~1-mL volume, and stored at −5 °C. A microcrystalline solid began to form within a few hours. After further storage at −20 °C (24 h), the solid was filtered from solution and washed with 9:1 diethyl ether/hexanes (20 mL), followed by air drying (0.035 g, 27% yield). Spin quantitation of a DMSO solution of known concentration against a $K_2(SO_3)_2NO$ standard (in $N_2$-purged 0.05 M $K_2CO_3$) indicated that the solid contained no solvents of crystallization. $^1H$ NMR (in $CDCl_3$, after reduction with $Na_2S_2O_4$): $\delta$ 7.23, 7.00 (8 H, q, aromatic), 6.85 (1 H, t, aromatic), 6.19 (1 H, s, NH), 3.01 (6 H, s, $NCH_3$). Mass spectrum (EI): *m/e* (relative intensity) 255 ($M^+$ − 15, 36), 226 (7), 210 (100), 181 (89), 167 (25), 154 (17). Anal. Calcd for $C_{15}H_{16}N_3O_2$: C, 66.65; H, 5.97; N, 15.54. Found: C, 64.29; H, 5.87; N, 15.54.

**PSII Sample Preparation.** PSII membranes were isolated from spinach leaves by a modified version[16] of the method of Berthold et al.[17] Membranes were then resuspended in buffer (20 mM MES/NaOH, pH 6.0, 15 mM NaCl, 5 mM $CaCl_2$, 30% (v/v) ethylene glycol). $O_2$ evolution activities of membranes were in the range of 400 μmol of $O_2$/((mg of chl)·h) when measured by a Clarke-type electrode. Samples (6–7 mg of chl/mL; 30–40 μM PSII) for controlled turnover experiments were prepared as follows: After loading of samples into quartz EPR tubes, samples were stored in an ice/water bath overnight (~14–17 h) to ensure

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full dark adaption. Samples were then treated in the dark with enough stock solution of $K_3Fe(CN)_6$ to bring the concentration to 500 μM, and incubated in the dark for about 0.5 h at 273 K. Samples were then illuminated for 3 min at 195 K and then dark adapted at 273 K to prepare samples in as pure a $S_1Y_D^{\bullet}$ state as possible.[18] A desired amount of a stock solution of herbicide in neat DMSO was then added in complete darkness to bring the herbicide concentration to the required amount. A large excess was generally used (~200 μM herbicide). Samples for power saturation studies on bound herbicide consisted of 28 μM PSII in standard buffer (no ferricyanide present, due to its effect on power saturation behavior) and 16 μM herbicide. The herbicide standard for power saturation studies consisted of 107 μM **2** in 30% ethylene glycol/water.

**Enzyme Activity Assays.** Enzyme activity assays for determination of $O_2$ evolution activity, $pI_{50}$ values of the herbicides, and herbicide binding constants were performed using a Clarke-type electrode as in ref 16. The assay buffer contained 20 mM MES/NaOH, pH 6.0, 10 mM NaCl, 5 mM $CaCl_2$, 1 mM $K_3Fe(CN)_6$, and 250 μM DCBQ, except for kinetic studies, where herbicide was the only electron acceptor present. For kinetic studies, $O_2$ evolution activity determinations were made three times for each set of concentrations used, for determination of errors. For other studies, generally two determinations per set were used. Kinetic data were fit directly to the Briggs-Haldane version of the Michaelis-Menten expression using an error-weighted nonlinear fit.

**Electron Paramagnetic Resonance Studies.** The following spectrometer conditions were used: microwave frequency, 9.1 GHz; magnetic field modulation frequency, 100 kHz. For measurement of the spectra of radical species, the following conditions were used: microwave power, $2.3 \times 10^{-8}$ W; magnetic field modulation amplitude, 4 G; digitization, ~0.4 G/point (512 points/scan); temperature, 15 K. For measurement of $S_2$-state multiline spectra, the following conditions were used: microwave power, $1.8 \times 10^{-5}$ W; magnetic field modulation amplitude, 20 G; digitization, ~2 G/point (1024 points/scan); temperature, 7 K.

PSII concentration quantitations were performed by comparison of a fully induced $Y_D^{\bullet}$ spectrum to the spectrum of a spin standard, either $K_2(SO_3)_2NO$ in $N_2$-purged 0.05 M $K_2CO_3$ or herbicide stock solution in neat toluene.

In PSII turnover control experiments, sample illuminations were performed at 195 or 273 K by immersion of the sample in a clear dewar containing either dry ice/acetone or ice/water, respectively. Illuminations at 250 K were performed using a cooled nitrogen flow standardized by thermocouple to a $CCl_4$ liquid-solid slurry. $S_2$-state multiline signal intensities were determined as peak-to-trough heights, using the four most intense peaks above and below $g = 2$, followed by normalization to the maximum multiline signal intensity for that sample. Nitroxide intensities were estimated from the integrated intensity of the $A_x$ line of the nitroxide radical, as this turning point does not overlap the $Y_D^{\bullet}$ spectrum. These intensities were converted to the full integrated intensity of the nitroxide radical by comparison to a standard radical spectrum and then were subtracted from the full integrated intensity of the nitroxide-containing PSII sample in the radical region. This difference, which should represent the intensity of $Y_D^{\bullet}$, was used as a correction factor for the nitroxide intensities, on the assumption that, after the 250 K illuminations, $Y_D^{\bullet}$ was fully induced, so that fluctuations in the difference value were due to instrumental instabilities. Lastly, it was found that $Y_D$ was oxidized in ~85% of the centers as initially prepared, prior to the addition of nitroxide radical.

Microwave power saturation experiments were conducted at 15 K. Nitroxide signal intensities for the herbicide standard and bound nitroxide signal intensities were determined using the peak-to-base-line height of the $A_x$ line of the radical, which, as the highest-field turning point of the radical, does not overlap any of the biological radicals.

**Optical Spectroscopy.** Optical spectroscopy measurements were performed at 25 °C on a flash-detection spectrophotometer similar to that described by Joliot et al.[19]

## Results

**Herbicide Design Strategies.** Two strategies were employed in the design of the desired molecules. In the first approach, a molecule in which the PSII-binding functionality is tethered to a separate electron-accepting group was considered. A potent binding group for the $Q_B$ site is the dimethylphenylurea moiety, and this group is the parent structure for a class of urea-based redox-inactive herbicides which act to terminate the electron-transfer chain by binding at the $Q_B$ site.[20] This group features high binding constants at the $Q_B$ site and an easily derivatizable aromatic ring. As an electron-accepting group, the nitroxide free radical moiety was chosen due to its similarity in reduction chemistry to the natural quinone electron acceptor and its one-electron redox chemistry.[21] That is, the nitroxide group participates in proton-coupled electron transfer in its reduction to the hydroxylamine, as do the secondary quinone electron acceptors, which are reduced to the dihydroxy form during PSII turnover. Linkages between the two are readily available through sulfonamide bridges; the coupling of the 4-sulfonyl chloride substituted dimethylphenylurea to 4-amino-TEMPO produced the dimethylphenylurea-TEMPO conjugate (**1**) (Scheme IV).

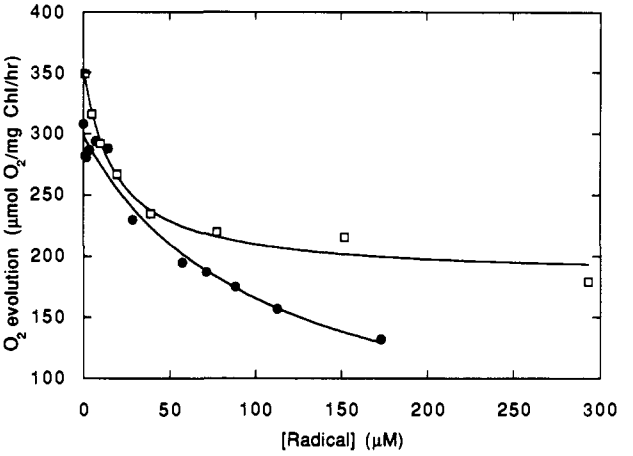
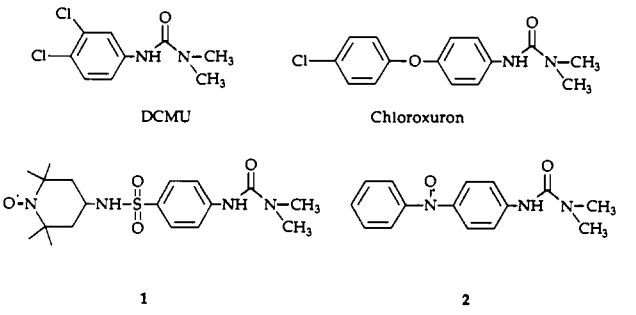



**Figure 1.** Inhibition of $O_2$ evolution activity of PSII by **1** (□) and **2** (●). Assay conditions are described in the Experimental Section.

**Scheme IV**




Since it was unknown whether the tethered electron acceptor would affect the binding of the urea group, a second approach was also employed. This involved the modification of a known herbicide structure to include an electron-accepting group with relatively minor perturbation of the overall herbicide structure. A search of the relevant literature quickly led to the herbicide chloroxuron [3-[4-(4-chlorophenoxy)phenyl]-1,1-dimethylurea; Scheme IV]. This urea derivative contains a 4-phenoxy group substituted on the dimethylurea fragment, the oxygen linkage of which could be converted to a nitroxide bridge via a nitroxide-forming reduction of a nitro-substituted phenyldimethylurea by Grignard reagent. This approach (with deletion of the chloride substituent of chloroxuron for synthetic simplification) produced (dimethylureido)phenyl phenyl nitroxide (**2**) (Scheme IV).

**$O_2$ Evolution Activity Studies.** Initial binding studies of herbicides **1** and **2** consisted of $O_2$ evolution activity inhibition experiments in the presence of the electron acceptor DCBQ (with ferricyanide present to ensure full oxidation of the DCBQ pool, Figure 1). **1** proved to bind less efficiently than **2**, with $pI_{50}$ values

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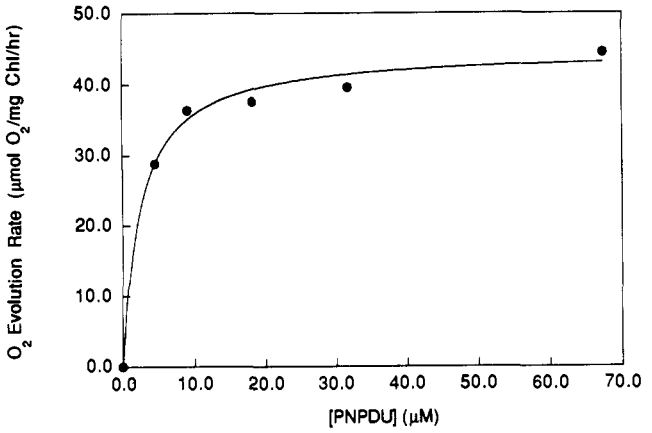


**Figure 2.** $O_2$ evolution rate as a function of the concentration of **2** (●). The curve is based on nonlinear least-squares fits to the Michaelis–Menten expression, weighted by experimentally determined errors.

of ~3.5 and ~3.9, respectively. We note that these $pI_{50}$ values are only mutually comparable, and not directly comparable to those for other herbicides, as part of the $O_2$ evolution observed in these experiments is due to electron transfer to the herbicide. Because of the higher $pI_{50}$ of **2**, implying a lower dissociation constant, this herbicide became the focus of continued study. $O_2$ evolution rates with **2** were studied when the redox-active herbicide was the only added electron acceptor. Maximal $O_2$ evolution rates with herbicide **2** proved to be about one-tenth of the maximal rate in the presence of quinone electron acceptors. The $O_2$ evolution rate as a function of herbicide concentration is presented in Figure 2. The data were fit to the Michaelis–Menten expression; the fit produced a Michaelis constant of $K_m = 2.5 \pm 0.3$ μM.

**Herbicide Radical EPR Microwave Power Saturation Studies.** The next element in the investigation of the interaction of **2** with PSII was an EPR microwave power saturation study of the herbicide in the bound and the unbound state. Power saturation data for the unbound, frozen solution state of the herbicide were fit (Figure 3) as previously described,[22] using the empirical function

$$\log\left(\frac{S}{\sqrt{P}}\right) = \left[K - \frac{b}{2}\log\left(1 + \frac{P}{P_{1/2}}\right)\right] \qquad (1)$$

where $S$ is the signal intensity, $P$ is the microwave power, $P_{1/2}$ is the power at half-saturation, $b$ is an inhomogeneity parameter, and $K$ is a proportionality factor. For the fits presented here, $b$ was fixed at a value of 1; $K$ and $P_{1/2}$ were allowed to vary. Nonlinear fitting of the data for the unbound herbicide produced a $P_{1/2}$ value of $(4.3 \pm 0.3) \times 10^{-7}$ W.

The power saturation data for the bound herbicide could not be fit adequately by eq 1, either by using the value of $P_{1/2}$ from the free herbicide or by allowing both $P_{1/2}$ and $K$ to vary (dashed line in Figure 3b), as expected, since there should be two contributions to this curve: one from unbound herbicide and one from herbicide bound in the $Q_B$ site, close enough for interaction with the non-heme iron(II) of PSII. These data were fit, therefore, by eq 2, which represents power saturation behavior as a sum of two terms identical to that in eq 1, each with a fractional prefactor:

$$\log\left(\frac{S}{\sqrt{P}}\right) = (n)\left[K^f - \frac{1}{2}\log\left(1 + \frac{P}{P^f_{1/2}}\right)\right] + (1-n)\left[K^b - \frac{1}{2}\log\left(1 + \frac{P}{P^b_{1/2}}\right)\right] \qquad (2)$$

where $n$ is the fraction of herbicide in the free state and the superscripts f and b denote parameters for the free and bound states of the herbicide. Fits of the data for bound herbicide were

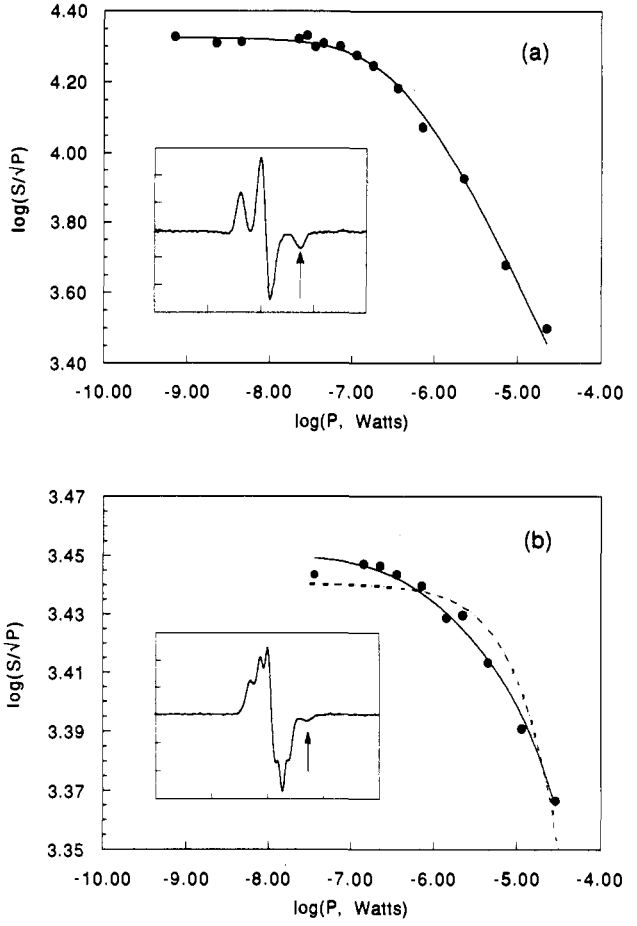


**Figure 3.** Microwave power saturation plots of (a) **2** (77 μM) in DMSO/ethylene glycol/water (1:10:35) fit to eq 1 and (b) **2** (16 μM) in the presence of PSII membranes (~28 μM) fit to eq 2. The dotted line in b represents a least-squares fit of the data to eq 1. Insets display typical spectra in each series with the $A_x$ peak used for signal intensity indicated by an arrow. Least-squares-fit parameters: (a) (eq 1) $K = (4.32 \pm 0.01)$, $P_{1/2} = (4.3 \pm 0.3) \times 10^{-7}$ W; (b) (eq 2) $K = (3.40 \pm 0.01)$, $P^b_{1/2} = (1.6 \pm 0.4) \times 10^{-4}$ W, $n = (0.057 \pm 0.009)$; (eq 1) $K = (3.44 \pm 0.01)$, $P_{1/2} = (6.2 \pm 0.8) \times 10^{-5}$ W. Experimental conditions: Spectra were recorded at 15 K using 4-G field modulation amplitude and 100-kHz field modulation frequency in the range 3180–3380 G with a digitization of 2.6 points/G. Microwave power values below $3.61 \times 10^{-8}$ W (log $P = -7.44$) produced spectra with $A_x$ intensities too low for accurate quantitation in the sample used in b due to the lower nitroxide concentration and overlap of the EPR signal from the stable tyrosine radical in PSII.

performed allowing only the values of $n$, $K^b$, and $P^b_{1/2}$ to vary; the parameters for the free herbicide term were fixed at the values determined from the previous fit to eq 1 for free herbicide. This fit (solid line in Figure 3b) indicated that $P^b_{1/2} = (1.6 \pm 0.4) \times 10^{-4}$ W, or a value about 370 times greater than that of the free herbicide; the increase in $P_{1/2}$ upon binding to PSII (attributed to interaction of the radical with the non-heme iron center) is further confirmation that the herbicide binds in the $Q_B$ site. The effect of the non-heme iron on the nitroxyl of the bound herbicide is less than that observed for a semiquinone bound at the $Q_B$ site, indicating that the nitroxide spin is located somewhat farther from the non-heme iron than the spin of the semiquinone. The fit also produced a value of $n = 0.057$ for the fraction of free herbicide. This value, when converted to a concentration by comparison of the $A_x$ peak of the herbicide to a standard, gives estimates of the bound and free concentrations of herbicide (assuming that the $A_x$ peak intensities are about the same for both bound and free herbicide). These can be used to estimate a dissociation constant of ~2 μM for **2**, using a simple 1:1 binding model; this value is essentially the same as that found for $K_m$. The closeness of these two values suggests that the dissociation rate constant for product hydroxylamine is small compared to the dissociation rate constant

(22) Innes, J. B.; Brudvig, G. W. *Biochemistry* **1989**, *28*, 1116–1125.

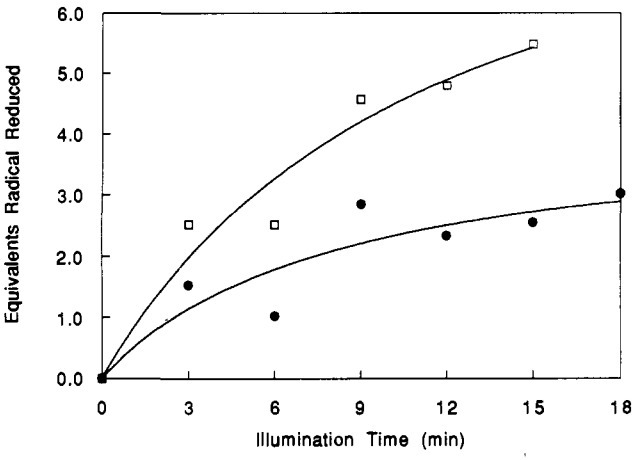


Figure 4. Reduction of 1 (□) and 2 (●) by PSII membranes as a function of illumination time at 273 K.

for nitroxide-containing herbicide.

**Photoreduction of Herbicide by PSII.** To verify that herbicides 1 and 2 participate in photoreduction chemistry with PSII, reaction centers (10 μM) were treated with ~500 μM 1 or ~150 μM 2. The samples were then illuminated at 0 °C for 3-min intervals, and EPR spectra were measured at the end of each interval. The change in the intensity of the total radical EPR signal was then compared to a spin standard to obtain the change in nitroxyl radical concentration; this value was compared to the PSII concentration (as measured by $Y_D^{\bullet}$ quantitation), giving the equivalents of nitroxyl radical reduced as a function of time. The data, presented in Figure 4, have been fit to simple saturation curves to obtain estimates of the number of nitroxyl radical equivalents reduced at saturation. These fits give values of about 10 nitroxyl radical equiv reduced/PSII center for 1 and about 4 equiv reduced/PSII center for 2, corresponding to quantitative reduction of the herbicide present in each case.

**Optical Spectroscopy Measurements.** Single-flash fluorescence measurements on PSII membranes with herbicide 2 bound exhibit an increase in the $Q_A^-$ fluorescence decay half-time; thus, herbicide binding impedes electron transfer from $Q_A$ to $Q_B$ under single-flash conditions, confirming binding at the $Q_B$ site. Multiple-flash experiments at 25 °C indicate that herbicide exchange takes place on a ~600-ms time scale. This corresponds to a sufficiently rapid exchange for reduction of multiple herbicide equivalents during continuous illumination at 0 °C, as is observed by EPR (Figure 4). Finally, the half-time for electron transfer to herbicide 2 (measured by fluorescence decay) is ~70 ms at 25 °C (data not shown). This may be compared to the half-time for electron transfer from $Q_A$ to $Q_B$ at the same temperature,[23] which has been measured at 0.1–0.2 ms in chloroplasts and 3 ms for the samples used in this study.

**Turnover Control Experiments.** After characterization of the binding of the herbicides to the $Q_B$ site of PSII and verification of their ability to accept electrons from the reaction center, turnover control experiments were conducted with the tighter-binding herbicide 2.

Since both EPR and optical studies showed that multiple turnovers occur at temperatures ≥0 °C, turnover control trials were performed at 250 K, where herbicide exchange should be slow. Samples for turnover control were prepared as follows: PSII centers were dark adapted for >10 h and then were treated with ferricyanide for oxidation of cytochrome $b_{559}$. Samples were then illuminated at 195 K for 3 min to fully induce the $S_2$ state. They were then allowed to dark adapt at 0 °C for ca. 20 min to allow the $S_2$ state to oxidize $Y_D$ or $Q_A^-$, forming a sample essentially completely in the $S_1$ state.[18] Herbicide 2 was then added (in complete darkness to avoid premature oxidation of the $S_1$ state) at a level of about 200 μM (nearly all $Q_B$ sites should be filled

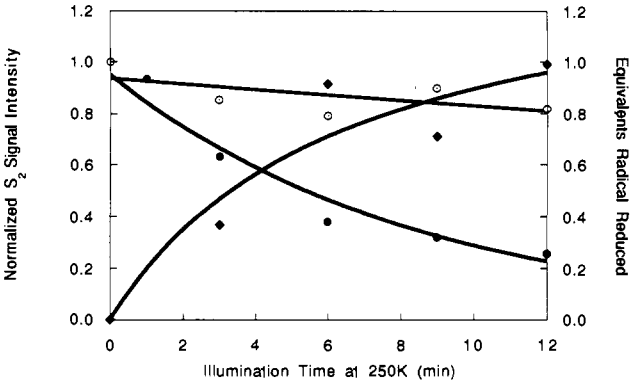


Figure 5. Time course of turnover control of PSII using herbicide 2. The decrement of the $S_2$ multiline EPR signal (●) follows the increment of the equivalents of 2 reduced (◆) as a function of illumination time at 250 K. A DCMU control experiment (⊙) is included for comparison.

at this concentration). After a short equilibration at 0 °C, a base-line spectrum was obtained, and samples were subjected to 3 min of illumination at 195 K for full induction of $S_2$ and formation of $Q_A^-$. Under these conditions, no electrons are transferred to bound herbicide radical and neither $Q_A^-$ oxidation nor the $S_2 \rightarrow S_3$[11,12] transition occurs. The sample was then continuously illuminated at 250 K for 3-min intervals, with spectra measured at the end of each interval. This temperature is sufficiently high for all S-state transitions to occur[12] and for the reduction of herbicide radical, but sufficiently low that exchange of bound herbicide should be slow. Quantitations of the reduction of herbicide radical and the disappearance of the $S_2$ multiline EPR signal which occur during the course of 250 K illumination appear in Figure 5. About one radical is reduced per reaction center, while about three-quarters of the $S_2$ EPR signal disappears. In addition, a final illumination at 195 K was performed after the last 250 K illumination; in no case was there a significant difference between the $S_2$ EPR signal intensity observed in this spectrum and that of the spectrum taken after the final 250 K illumination. Finally, performance of the protocol described here using the redox-inactive urea herbicide DCMU shows only a slight dropoff in $S_2$ EPR signal intensity during the course of the illuminations, also shown in Figure 5.

## Discussion

We interpret the results described above as indicating that herbicide radicals 1 and 2 bind at the $Q_B$ binding site of PSII, with the reported estimated dissociation constants. This conclusion is supported by the following lines of evidence. First, the $Q_B$ site is the known binding location of urea-type herbicides in PSII;[20] likewise, quinone-competitive herbicides are known to bind at this site in the bacterial photosynthetic reaction center.[24] Next, the increase in $P_{1/2}$ of herbicide 2 upon binding is taken to confirm this location, as the $Q_B$ site is within a few angstroms of the non-heme iron center. Proximity to the non-heme iron enhances relaxation of the nitroxyl radical (and thus increases the microwave power required for saturation) via, in part, a dipole–dipole coupling mechanism.[25] The relaxation effects of the non-heme iron on 2 are smaller than those observed for semiquinones bound in the $Q_B$ site. Such an effect is reasonable in light of crystallographic characterization of herbicides bound to the bacterial reaction center: while *o*-phenanthroline hydrogen bonds to an imidazole ligand of the non-heme iron, as do quinones, the herbicide terbutryn binds in the $Q_B$ site but substantially farther from the non-heme iron (no high-quality structural data are available for DCMU binding to the bacterial reaction center).[24] Even if urea-based herbicides do have binding modes similar to those of quinones to PSII, the unpaired electron density in our redox-active herbicides will still be several angstroms removed from the

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analogous location in quinones, as the nitroxide unit in both **1** and **2** is separated from the urea-based binding portion of the herbicide. Finally, we note that the fluorescence studies are also consistent with binding in the $Q_B$ site.

Upon continuous illumination of herbicide-treated PSII at 250 K, we conclude that roughly 75% of the PSII centers advance by one S state from $S_2$ to $S_3$ and that $Y_D$ is oxidized in competition with the $S_2 \rightarrow S_3$ transition in most of the remaining PSII centers, on the basis of the following considerations:

(1) Only one nitroxide radical is reduced per PSII center. Together with the one electron accepted by $Q_A$, this allows 2 oxidizing equiv to be formed on the donor side of PSII. One of these is accounted for by the $S_1 \rightarrow S_2$ transition which was fully induced by illumination at 195 K. The remaining oxidizing equivalent is mostly accounted for by oxidation of $S_2$ to $S_3$ in approximately 75% of the PSII centers and by oxidation of $Y_D$ in about 15% of the PSII centers.

(2) Illumination of samples at 195 K after the final 250 K illumination produces no change in $S_2$ EPR signal intensity from the previous (250 K) illumination. This indicates that there are no centers in $S_1$ which can be oxidized to $S_2$ in the samples. Untreated PSII centers which are continuously illuminated at temperatures of 235 K or above attain a distribution of S states,[14] so if this randomization process were occurring during the illumination protocol, there would be centers in the $S_1$ state which could advance to $S_2$ during the final 195 K illumination, resulting in a greater $S_2$-state multiline EPR signal yield. As there is no indication of this increase, we infer that significant randomization of S states does not occur, and the centers which have left $S_2$ are, therefore, in $S_3$. It is still logically possible that centers have nonrandomly piled into $S_0$ (the only remaining stable S state); we rely on the single equivalent of electrons reducing the herbicide to rule this possibility out.

(3) Performance of the illumination protocol using DCMU instead of herbicide **2** results in little change in $S_2$ EPR signal intensity. Thus, at 250 K, use of a redox-inactive herbicide allows only one charge separation to form, $S_1Q_A \rightarrow S_2Q_A^-$, and this state is stable under the temperature and illumination conditions used. This is consistent with our picture of the functioning of the redox-active herbicide under the experimental conditions described, in which an additional step is allowed due to the ability of the herbicide to accept 1 equiv of electrons:

$$S_1Q_AN^{\bullet}O \longrightarrow S_2Q_AN^{\bullet}O \xrightarrow[H^+]{} S_3Q_ANOH.$$

In this scheme, the herbicides function chemically just as do quinones, participating in a proton-coupled electron-transfer reaction. The requirement of temperatures in the range of 250 K or higher for this reaction is likely due, in part, to the need for proton transfer during the reduction.

We attribute the incomplete conversion of $S_2$ to $S_3$ to the oxidation of competing electron donors. Since cytochrome $b_{559}$ has been chemically oxidized, the only available electron donors are $Y_D$ and chlorophyll (see Scheme I). It is known to be difficult simultaneously to ensure a homogeneous initial $S_1$ state and the full oxidation of $Y_D$ (as mentioned above, we determined that $Y_D^{\bullet}$ was ~85% induced in the sample before **2** was added). We have chosen to prepare a sample with a homogeneous $S_1$ state at the expense of $Y_D$ oxidation in order to facilitate characterization of the product S state. Therefore, approximately 0.15 equiv of $Y_D$ is oxidized during the experiment; the remainder of the missing electron donor equivalent (0.10 equiv) is due to chlorophyll oxidation, to some reduction of $Y_D^{\bullet}$ upon adding **2**, or to additional S-state advancement. Also, some of the remaining fraction may be within the error of the measurement.

Previous attempts to produce high yields of the $S_3$ state have encountered the same barrier to complete one-electron advancement beyond $S_2$,[13] and it appears that this is a fundamental limitation of the electron-donor branching ratio in PSII. The chief advantage of our approach to the problem of $S_3$ formation is that, while it cannot overcome the electron-donor structure of PSII, it does allow preparation of the $S_3$ state in high yields in samples that are much more concentrated than those previously employed. Thus, the net production of $S_3$ is greater using the turnover-control method described here than with previous methods. Preparations of highly concentrated samples enriched in the $S_3$ state should facilitate spectroscopic study of this important intermediate in the water-oxidation reaction of photosystem II.

It is clear from this initial study that this work will benefit from the design of tighter-binding redox-active herbicides; investigation of the binding constraints of the $Q_B$ site with regard to electron-acceptor structure should lead to higher herbicide binding constants.

The approach to turnover control described here is expected to yield results in a variety of areas of PSII research, in addition to facilitating spectroscopic study of the $S_3$ state. First, chemical and ligand-binding studies of the $S_3$ state will benefit. In addition, the ability to limit the reaction center to two turnovers should find application in the study of the assembly of the tetramanganese cluster, and may allow formation and observation of a dinuclear Mn(II)–Mn(III) intermediate. No intermediates in this process have been detected previously, and cluster assembly in the presence of herbicide radical may allow the formation of a sufficient yield of an intermediate for study of such a species. Next, a two-electron-accepting version of the herbicides described here (if electron transfer could be made to proceed at a sufficiently low temperature) could provide a route to preparation of the unstable $S_4$ state, which is potentially EPR-active. A key to this may be the use of electron acceptors which do not require protonation, so that electron transfer can proceed at temperatures where proton transfer does not occur. Finally, this general approach may benefit the study of other multiple-turnover redox enzymes for which a suitable redox-active substrate can be designed.

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