Photoinactivation of Photosystem II by *in Situ*-Photoproduced Hydroxyurea Radicals†

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ABSTRACT: Oxygenic photosystem (PS) II complex from spinach photooxidized hydroxyurea (HU) to produce its aminoxy radical, which was identified by its electron spin resonance spectrum. HU was apparently photooxidized by the water-oxidizing enzyme (WOE) since the photooxidation reaction was blocked by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). HU radicals photoproduced by the WOE inhibited the electron transfer between the redox-active tyrosine residue (Y_Z), which is involved in electron transfer from the WOE to the reaction center chlorophyll of PS II, and the secondary quinone electron acceptor. Treatment of PS II complex with Tris resulted in the appearance of a CCCP-insensitive photooxidation site for HU. Photoproduced HU radicals in oxygenic and Tris-treated PS II complex decayed with first-order kinetics, an indication that the radicals reacted primarily with surrounding molecules rather than decayed through spontaneous dismutation or recombination. HU inhibited the diphenylcarbazide-supported photoreduction of 2,6-dichlorophenolindophenol (DCIP) in Tris-treated PS II complex preincubated only under illumination, but this inhibition was suppressed when ascorbate was added to scavenge HU radicals. If examined in darkness, HU radicals could not, however, inhibit subsequent photoreduction of DCIP. Therefore, the photoproduced HU radicals interact with a photogenerated site(s) in the PS II complex. The photoproduction of $Y_Z^•$, a radical of Y_Z, was suppressed to about 40% in Tris-treated PS II complex by the *in situ*-photogenerated HU radicals, and the yield of a cation radical of chlorophyll, close to the PS II reaction center, was increased, while the production of a radical of another redox-active tyrosine residue in PS II ($Y_D^•$) was hardly affected. This indicates that the HU radicals modified $Y_Z^•$ rather than $Y_D^•$.

Oxygenic photosystem (PS)[1] II complex in thylakoid membrane, which catalyzes the light-driven oxidation of water and the reduction of plastoquinone, is composed of membrane-spanning and extrinsic polypeptides, as well as inorganic cofactors such as Mn, Ca^{2+}, Cl^-, and Fe^{2+} [for review, see Debus (1992)]. On the donor side of the PS II reaction center, two redox-active tyrosine residues have been identified. Tyr-161 of the D1 polypeptide (Y_Z; Debus et al., 1988b; Metz et al., 1989) is oxidized by $P680^+$ to form its radical ($Y_Z^•$), which subsequently oxidizes the Mn cluster in the water-oxidizing enzyme (WOE). Another redox-active residue, Tyr-160 [Tyr-161 in spinach (Svensson et al., 1991)], of the D2 polypeptide (Y_D; Debus et al., 1988a; Vermaas et al., 1988) also forms a radical ($Y_D^•$), but the function of this radical remains unknown. A close similarity in terms of microenvironment around these two tyrosine residues in the backbones of their respective proteins has been suggested from (1) the similarities between the electron spin resonance (ESR) spectra of $Y_Z^•$ and $Y_D^•$ (Barry et al., 1990), (2) the conserved amino acid residues adjacent to the tyrosine residues (Babcock et al., 1989), and (3) the symmetrical arrangement of the tyrosine residues relative to P680 (Svensson et al., 1990). $Y_Z^•$ and $Y_D^•$ can be distinguished from each other by their decay kinetics. $Y_Z^•$ is detectable by ESR as signal $II_{very\ fast}$ ($t_{1/2}$ = 0.4–0.9 ms) in intact chloroplasts and algae (Warden et al., 1976) and as signal II_{fast} ($t_{1/2}$ = 0.5 s) in Tris-treated thylakoids (Babcock & Sauer, 1975). $Y_D^•$, by contrast, decays slowly ($t_{1/2} \approx$ 5–40 and 100–500 min by biphase) in the PS II membranes (Vass & Styring, 1991) and, hence, has been referred to as signal II_{slow}. The differences in kinetics are attributable at least in part to differences in the distances to the tyrosine residues from the Mn cluster or from His-190s in the D1 and D2 polypeptides. The former distances are estimated to be about 7 Å for Y_Z (Svensson et al., 1991) and 30–40 Å for Y_D (Evelo et al., 1989), and the latter distances are predicted by modeling to be about 4 and 2–3 Å, respectively (Svensson et al., 1990). By contrast to Y_Z (Roffey et al., 1994), Y_D has been proposed to form a hydrogen bond to His-190 in the D2 polypeptide (Tang et al., 1993). This would also be responsible for the differences in the decay kinetics of the two tyrosine radicals.

In addition to $Y_Z^•$ and $Y_D^•$ in the PS II reaction center, tyrosine radicals have been found as catalytic intermediates in some enzymes, for example, ribonucleotide reductase (Reichard & Ehrenberg, 1983), galactose oxidase (Whittaker & Whittaker, 1990), and prostaglandin endoperoxide synthase (Karthein et al., 1988). Note, however, that the function of the tyrosine radical in the last enzyme is still the subject of some controversy (Smith et al., 1992). The stable tyrosine radical in ribonucleotide reductase has been well characterized [for review, see Stubbe (1989)] and has been studied in experiments with hydroxyurea (HU; Atkin et al., 1973; Berglund & Sjöberg, 1979; Kjøller Larsen et al., 1982; Sahlin et al., 1982), which is an inhibitor of DNA synthesis (Rosenkranz et al., 1966). It has been proposed that the tyrosine radical of the reductase abstracts the hydrogen at C-3 of the ribosyl moiety of the substrate, ribonucleoside

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DMBQ, 2,6-dimethylbenzoquinone; DPC, diphenylcarbazide; ESR, electron spin resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); HU, hydroxyurea; MES, 2-(*N*-morpholino)ethanesulfonic acid; P680, reaction center chlorophyll of PS II; $P680^+$, cation of P680; PS, photosystem; Q_B, secondary quinone electron acceptor; Tris, tris-(hydroxymethyl)aminomethane; WOE, water-oxidizing enzyme; Y_D, tyrosine-161 in the D2 polypeptide; Y_Z, tyrosine-161 in the D1 polypeptide.

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diphosphate, and it is regenerated by reduction of the reaction intermediate, a 3′-deoxynucleotide radical (Reichard & Ehrenberg, 1983; Stubbe, 1989). HU reduced the tyrosine radical without subsequent regeneration to consequently inhibit the catalytic activity (Lassmann et al., 1992; Karlsson et al., 1992). HU also inactivates ascorbate peroxidase by attacking the enzyme; the aminoxy radicals of HU are produced upon oxidation by an intermediate in the enzyme-catalyzed reaction with hydrogen peroxide (Chen & Asada, 1990a). The attack probably occurs at a tryptophan residue, which is the putative active site, corresponding to that of the homologous enzyme, cytochrome *c* peroxidase (Chen et al., 1992c; Kubo et al., 1992).

In the present study, we examined the reactivity of HU with the two tyrosine radicals $Y_Z^{\bullet}$ and $Y_D^{\bullet}$ in PS II membranes. We observed the univalent photooxidation of HU to its aminoxy radicals by WOE and $Y_Z^{\bullet}$. The generated HU radicals inhibited the oxidation of Y_Z by $P680^+$ but only slightly affected the level of the ESR signal from $Y_D^{\bullet}$. Part of this work has been presented in a preliminary form (Kawamoto et al., 1992).

MATERIALS AND METHODS

Preparation of Oxygenic and Tris-Treated PS II Membranes. Oxygenic PS II membranes were prepared from commerically purchased spinach leaves with Triton X-100, as described by Kuwabara and Murata (1982). The PS II membranes were suspended in buffer A (0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 20 mM MES-NaOH, pH 6.5) that contained 30% ethylene glycol and stored at −84 °C. Tris-treated PS II membranes were prepared by incubation of the oxygenic PS II membranes (0.5 mg of Chl mL^{-1}) in 0.8 M Tris-HCl, pH 9.0, for 30 min in darkness in an ice bath (Yamashita & Butler, 1969). The resultant Tris-treated PS II membranes were sedimented by centrifugation at 35000*g* for 10 min and suspended in buffer A at 2–4 mg of Chl mL^{-1}.

Assays of Oxygen-Evolving and DCIP-Photoreducing Activities. Oxygen-evolving activity of PS II membranes was measured in buffer B (0.4 M sucrose, 20 mM $CaCl_2$, and 50 mM HEPES-NaOH, pH 6.8) that contained 2 mM 2,6-dimethylbenzoquinone (DMBQ), under illumination of 1000 W m^{-2}, with a Clark-type oxygen electrode. Illumination was provided by actinic light from a tungsten lamp that had been filtered through water. Electron transport activity in Tris-treated PS II membranes was determined photometrically, at 610 nm, as the rate of photoreduction of 2,6-dichlorophenolindophenol (DCIP). The assay mixture (5 μg of Chl mL^{-1}) contained buffer C (0.1 M sucrose, 30 mM HEPES-NaOH, pH 7.5; Izawa, 1980) and 0.1 mM DPC as an electron donor or buffer D (0.8 M sucrose, 15 mM NaCl, and 50 mM MES-NaOH, pH 6.5) and 1 μM $MnCl_2$/3 mM hydrogen peroxide as electron donors (Blubaugh et al., 1991), with inclusion of 30 μM DCIP. For this assay, actinic light from a tungsten lamp was passed through a filter with a cutoff above 660 nm and the photomultiplier was protected by a 610-nm interference filter.

Recording of ESR Spectra. ESR spectra were recorded with a JES-RE2X ESR spectrometer (JEOL, Tokyo, Japan) at room temperature using a flat cell (thickness, 0.4 mm), which was illuminated with white actinic light from a tungsten lamp that was passed through a water filter.

Determination of Mn Content. PS II membranes that had been incubated with HU in darkness were diluted with buffer A. The suspension was centrifuged at 3000*g* for 10 min, and the resultant pellet was washed once more with the buffer. The concentration of manganese remaining in the pellet was determined with an atomic absorption spectrometer (Model 303; Hitachi, Tokyo, Japan) equipped with a graphite atomizer.

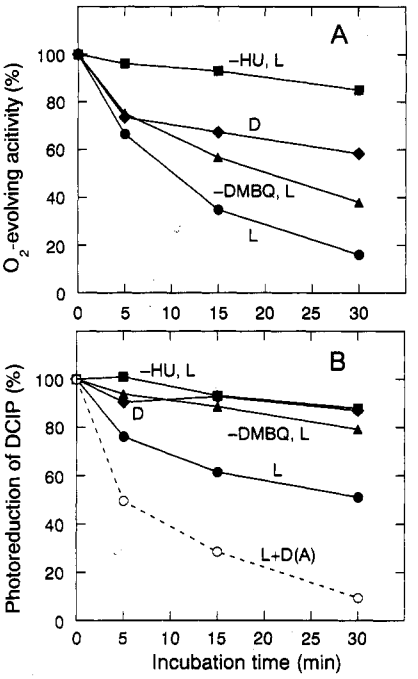

FIGURE 1: Inhibition of oxygen-evolving activity (A) and DPC-supported photoreduction of DCIP (B) of PS II membranes by hydroxyurea. Oxygenic PS II membranes (1 mg of Chl mL^{-1}) were incubated with 20 mM HU and 2 mM DMBQ in buffer A in darkness (D) or light (30 W m^{-2}) (L) in a cell (1 cm in diameter) at 25 °C with stirring. Where indicated, either HU or DMBQ was omitted. After incubation for indicated times, the incubation mixture was diluted 20-fold with buffer A and the PS II membranes were sedimented by centrifugation at 17500*g* for 10 min. After washing the resultant pellet once more with buffer A, the oxygen-evolving activity was determined as described in Materials and Methods (A). The DPC-supported photoreduction of DCIP was determined after treating the incubated samples by Tris buffer, as described in Materials and Methods (B). The data L+D(A) are obtained by addition of the decrease extent of the photoreduction of DCIP with HU and DMBQ in the light to that of the oxygen evolution in darkness.

Source of Enzyme. Horseradish peroxidase (EC 1.11.1.7) was obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Inhibition of the Oxygen Evolution by Hydroxyurea. The oxygen-evolving activity of PS II membranes was diminished with a half-time of about 2 h upon dark incubation with 20 mM HU in buffer A at 4 °C, and it was completely lost after a 4-h incubation. By the time the activity had disappeared, 80% of the total initial manganese had been released from oxygenic PS II membranes, as determined by atomic absorption spectrometry (Chen & Asada, 1990b). Thus, the dark inhibition by HU was mainly due to the release of manganese from the WOE as in the case of NH_2OH (Cheniae & Martin, 1971), NH_2NH_2 (Cheniae & Martin, 1971), and acetone hydrazone (Vass et al., 1990). However, a much longer incubation was necessary for the dark inactivation by HU than by NH_2OH [$t_{1/2} \approx 1$ min, Cheniae and Martin (1971)].

As compared with the results of dark incubation with HU, the oxygen-evolving activity of PS II membranes was more inhibited under illumination in the presences of HU and DMBQ. The photoinhibition of the oxygen evolution was suppressed if either HU or DMBQ was absent or in darkness (Figure 1A), an indication of acceleration of the photoinhibition by turnover of PS II. In order to examine whether HU

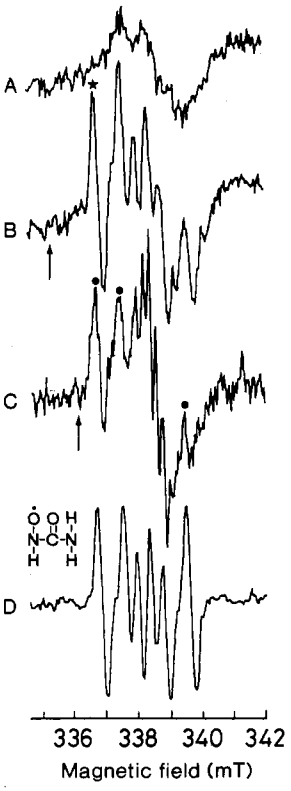

FIGURE 2: Photoproduction of hydroxyurea radicals by oxygenic (A, B) and Tris-treated (C) PS II membranes. The reaction mixture contained PS II membranes (1 mg of Chl mL^{-1}), 40 mM HU, and 2 mM DMBQ in buffer A in darkness (A) and under illumination (B, C). In trace D, HU radicals were produced by 40 mM HU and 10 mM hydrogen peroxide with 120 units mL^{-1} horseradish peroxidase in buffer A. ESR spectra were recorded as described in Materials and Methods. Scanning was started within 2 min after the addition of HU (A, B, C) or hydrogen peroxide (D). The light (75 W m^{-2} in trace B and 40 W m^{-2} in trace C) was turned on at the time indicated by the arrow. Instrument settings: microwave power, 10 mW; modulation amplitude, 0.32 mT; time constant, 0.1 s; scan speed, 7.5 mT min^{-1}; with the exception that the time constant and scan speed were 0.3 s and 3.75 mT min^{-1}, respectively, for the horseradish peroxidase system. The asterisk indicates 336.7 mT. The dots in trace C indicate peaks of the spectrum of HU radicals. The structural formula in trace D is that of the aminoxy radical of hydroxyurea.

inactivated WOE or inhibited the electron transport between Y_Z and the secondary quinone electron acceptor (Q_B), the oxygenic PS II membranes were treated by HU and light as in Figure 1A and the resultant PS II membranes were treated with 0.8 M Tris (pH 9.0) to remove the extrinsic proteins and the Mn cluster of WOE (Kuwabara & Murata, 1983). The DPC-supported photoreduction of DCIP was decreased after the treatment with HU and DMBQ under illumination but only slightly affected without either HU or DMBQ or in darkness (Figure 1B). Sum of the dark inhibition by HU through Mn release and the HU/light inhibition of the photoreduction of DCIP [Figure 1B, L+D(A)] was well consistent with the photoinhibition of the oxygen evolution in the presence of HU and DMBQ (Figure 1A, L). Therefore, the primary target site of light-dependent inhibition by HU in the oxygenic PS II membranes is between Y_Z and Q_B but not WOE.

Photoproduction of Hydroxyurea Radicals at the Water-Oxidizing Enzyme. On illumination of oxygenic PS II membranes in the presence of HU and DMBQ, we observed an ESR spectrum of the photooxidation product that was characterized by six lines centered at 338.3 and of 3.7 mT in overall spectral width (Figure 2B). In darkness, only the ESR spectrum of $Y_D^{\bullet}$ was observed (Figure 2A), as was also the case in the absence of HU (data not shown). The number and

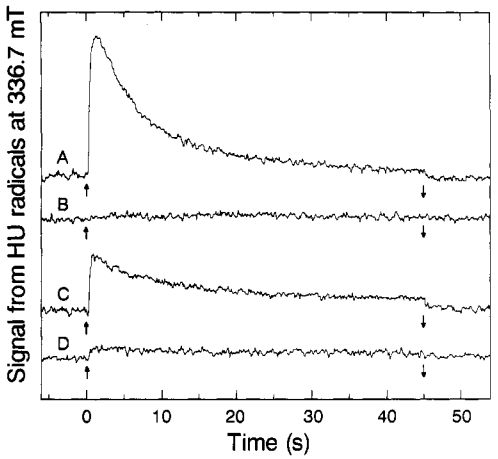

FIGURE 3: Time courses of the production and decay of hydroxyurea radicals upon illumination of PS II membranes. The reaction mixture contained oxygenic (A, B) or Tris-treated (C, D) PS II membranes (1 mg of Chl mL^{-1}) in buffer A including 40 mM HU and 2 nM DMBQ with the following additions: (B) 10 μM CCCP, (D) 4 mM DPC. The ESR signal was recorded as described in Materials and Methods. Instrument settings were the same as those described in the legend to Figure 2 with the exception that the time constant was 0.01 s and the magnetic field was fixed at 336.7 mT. The actinic light (280 W m^{-2}) was turned on at (↑) and off at (↓).

field positions of the peaks of the spectrum after subtraction of the background $Y_D^{\bullet}$ signal were equivalent to those of the spectrum of radicals generated from HU by a mixture of hydrogen peroxide and horseradish peroxidase (Figure 2D). This spectrum has been shown to be identical to a simulated spectrum of aminoxy radicals of HU when two hyperfine coupling constants, namely, a_N = 0.8 mT and a_H = 1.18 mT, are assumed (Chen & Asada, 1990a). Thus, HU was univalently photooxidized to its aminoxy radicals in the oxygenic PS II membranes. The acceleration of photoinhibition of the photoreduction of DCIP in the presence of HU (Figure 1B) seems, therefore, to be caused by photoproduced HU radicals.

The kinetics of photoproduction of HU radicals were monitored at 336.7 mT (Figure 3), where the signal of the HU radical did not overlap with that of $Y_D^{\bullet}$ (Figure 2). The ESR signal due to HU radicals increased rapidly at the onset of illumination (280 W m^{-2}), and the intensity of the signal reached a maximum within 1 s, which was followed by a decay with a half-time of 6.6 s (Figure 3A). Photoproduction of HU radicals was hardly observed in the absence of DMBQ or in the presence of 10 μM DCMU (data not shown). Thus, the rate of production of HU radicals was increased by an increase in the rate of turnover of PS II. When an ADRY (acceleration of the deactivation reactions of the water-splitting enzyme system) reagent, namely, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Renger, 1972), was present at 10 μM, the oxygen-evolving activity, with DMBQ as the electron acceptor, was inhibited by 85%. Under these conditions, the photooxidation of HU was completely inhibited even in the presence of the electron acceptor (Figure 3B). HU was, therefore, photooxidized at WOE in oxygenic PS II membranes, and the HU radicals generated in WOE inactivated the electron transfer from Y_Z to Q_B (Figure 1B).

Another Site for Photoproduction of Hydroxyurea Radicals in Tris-Treated PS II Membranes. The photoproduction of HU radicals was also observed in the Tris-treated PS II membranes (Figures 2C and 3C), where the three peripheral proteins and manganese of WOE were depleted (Kuwabara & Murata, 1983). The ESR spectrum of the molecular species that was photogenerated by Tris-treated PS II membranes was the same as that observed with oxygenic PS II membranes,

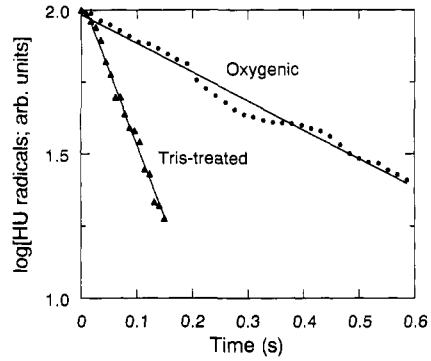

FIGURE 4: Decay of hydroxyurea radicals in darkness after illumination of oxygenic and Tris-treated PS II membranes. The experimental conditions were the same as those described in the legend to Figure 3A,C. Decay of HU radicals was monitored in darkness after illumination for 1.5 s. The data points were extracted from single-decay traces on the chart paper. The solid lines were obtained by the least squares on the assumption that HU radicals decayed with a pseudo-first-order reaction.

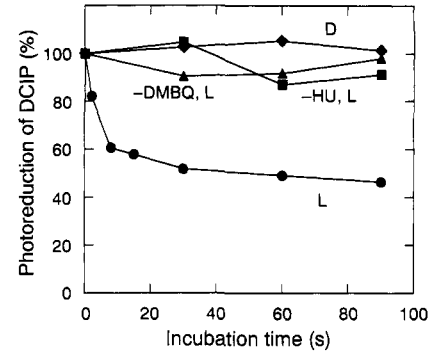

FIGURE 5: Effect of hydroxyurea on the transport of an electron from DPC to DCIP in Tris-treated PS II membranes. Tris-treated PS II membranes (1 mg of Chl mL^{-1}) were incubated in buffer A that contained 1 mM HU and 2 mM DMBQ. Where indicated, either HU or DMBQ was omitted. The mixture was incubated under white light (50 W m^{-2}; L) or in darkness (D) in a flat cell at room temperature. After incubation of the indicated time, the mixture was diluted 200-fold with buffer C for measurement of the rate of photoreduction of DCIP. The photoreduction of DCIP with 0.1 mM DPC was quantitated under illumination of 130 W m^{-2} as described in Materials and Methods.

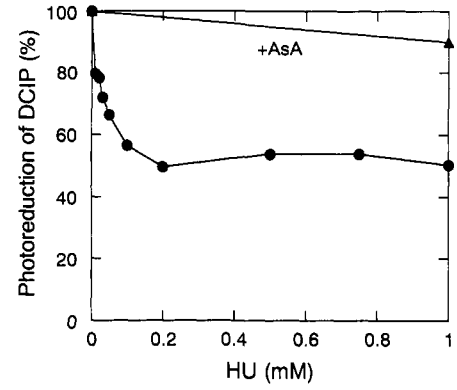

FIGURE 6: Dependence on the concentration of hydroxyurea of the photoreduction of DCIP supported by DPC and effect of ascorbate (AsA) on the inhibition of the DPC-supported photoreduction of DCIP (+AsA) by Tris-treated PS II membranes. The reaction mixture contained Tris-treated PS II membranes (1 mg of Chl mL^{-1}), 2 mM DMBQ, HU at indicated concentrations, and 1 mM ascorbate (+AsA) in buffer A. The mixture was preincubated under white light (50 W m^{-2}) for 30 s in a flat cell at room temperature. After the preincubation, the rate of photoreduction of DCIP was measured as described in the legend to Figure 5.

with the exception of an overlapping spectrum of semiquinone radicals of DMBQ centered at 338.7 mT (Figure 2C). The semiquinone radicals did not participate in the production of HU radicals because addition of potassium ferricyanide to quench the semiquinone radicals or alternation of the electron acceptor to DCIP or 2,5-dichloro-*p*-benzoquinone did not affect the photoproduction of HU radicals (data not shown). DPC largely suppressed the photoproduction of HU radicals with the Tris-treated PS II membranes (Figure 3D). Therefore, the HU radicals would be produced by the photooxidation of HU on the donor side of the PS II reaction center. The photooxidation of HU by Tris-treated PS II membranes was not inhibited by 10 μM CCCP, and the concentration of HU for half-maximum photoproduction of HU radicals was 3 mM with Tris-treated PS II membranes, whereas it was above 8 mM with the oxygenic PS II membranes. Thus, a new site for photooxidation of HU appeared in Tris-treated PS II membranes upon removal of the Mn cluster and the peripheral proteins of the WOE.

Hydroxyurea Radicals React with Surrounding Sites on PS II Membranes. The level of the photogenerated HU radicals rapidly decreased after reaching a maximum with both oxygenic and Tris-treated PS II membranes (Figure 3). This suggests either that the rate of photoproduction of HU radicals was decreased during the illumination or that the HU radicals rapidly disappeared by interaction with surrounding sites in PS II. The photoproduced HU radicals decayed with first-order kinetics after termination of illumination (Figure 4), a result that suggests that the photoproduced HU radicals were quenched through interactions with surrounding sites on PS II membranes but not by spontaneous dismutation or recombination. Such interaction would result in loss of the activities of PS II membranes, as indicated in Figures 1 and 5, with consequent decrease of the rate of photoproduction of HU radicals. Photoproduced HU radicals decreased more rapidly with Tris-treated PS II membranes ($t_{1/2} \approx 0.07$ s) than with the oxygenic PS II membranes ($t_{1/2} \approx 0.28$ s; Figure 4). This suggests that the photoproduced HU radicals interacted with different sites on each sample or, alternatively, that the HU radicals were quenched by the same component in both samples but the quenching was facilitated by the removal of the Mn cluster and the peripheral proteins of the WOE.

Hydroxyurea Radicals Inactivate Tris-Treated PS II Membranes. The activity of DPC-supported photoreduction of DCIP by Tris-treated PS II membranes was decreased rapidly in the fast phase by illumination in the presences of HU and DMBQ. The activity was decreased to about 60% for the 8-s illumination, but further inactivation was very slow during prolonged illumination (Figure 5). The HU-caused photoinactivation of the Tris-treated PS II membranes was very rapid as compared with that of the oxygenic PS II membranes (Figure 1). Little inactivation was observed when either HU or DMBQ was omitted from the preincubation mixture or in darkness. Thus, inactivation was observed only when PS II turned over in the presence of HU, and it was probably caused by interaction of the photoproduced HU radicals with a component(s) in PS II.

The photoinactivation by HU was saturated at 0.2 mM, which was at least by 2 orders of magnitude lower than that for the oxygenic PS II membranes, and further inactivation was not observed upon increases in the concentration of HU (Figure 6). The inactivation was irreversible since the activity was not restored by dilution of HU in the treated samples to 5 μM, at which the photoreduction of DCIP was unaffected (data not shown).

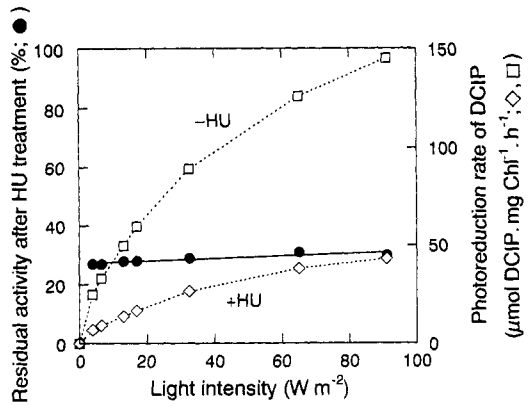

FIGURE 7: Effect of hydroxyurea on the manganese- and hydrogen peroxide-supported photoreduction of DCIP in Tris-treated PS II membranes. Tris-treated PS II membranes were incubated with (open diamond) or without (open square) 0.5 mM HU as described in the legend to Figure 6. After the incubation, the mixture was diluted 200-fold with buffer D. The Mn^{2+}/H_2O_2-supported photoreduction of DCIP was monitored at the indicated intensities of actinic light as described in Materials and Methods. The closed circles represent the residual activity of the Tris-treated PS II membranes at various intensities of the actinic light after the treatment with HU and light.

After the treatment of the Tris-treated PS II membranes with HU under 30-s illumination, the activity was determined at various intensities of light (Figure 7). The inactivation ratio was not affected by the light intensity, indicating that the inhibition site is the step of photoreaction of PS II but not the step of the dark reaction. About 30% of the residual activity was observed even when the HU concentration (0.5 mM) was high enough to saturate the inactivation. It has been shown that the Mn^{2+}/H_2O_2 system donates an electron to $P680^+$ only via Y_Z (Chen et al., 1992a), but DPC can donate an electron to a site other than $Y_Z^\bullet$ (Blubaugh & Cheniae, 1990). Thus, the activity of electron transport through Y_Z was not completely lost by HU even at saturating concentration.

Hydroxyurea Radicals React with Photogenerated Species of PS II Component(s). The photoinhibition by HU of the DPC-supported photoreduction of DCIP in the Tris-treated PS II membranes was suppressed by ascorbate (Figure 6), which is oxidized by aminoxy radicals of HU to produce monodehydroascorbate radicals (Chen & Asada, 1990a). HU radicals photoproduced by Tris-treated PS II membranes were quenched by ascorbate, and the ESR signal of monodehydroascorbate radicals appeared at the same time (data not shown). This observation indicates that the HU radicals are the molecular species that participate in the photoinactivation.

To examine whether HU radicals interact with PS II components and induce their inactivation in darkness, HU radicals were generated by hydrogen peroxide and horseradish peroxidase. Inhibition of the DPC-supported photoreduction of DCIP was not observed when Tris-treated PS II membranes (1 mg of Chl mL^{-1}) where incubated in buffer A that contained 20 mM HU, 0.5 mM hydrogen peroxide, and 90 units of horseradish peroxidase mL^{-1} for 100 s in darkness. The concentration of HU radicals produced by the hydrogen peroxide and horseradish peroxidase, as measured by ESR, was about 4.5-fold higher than the maximum concentration of the HU radicals photoproduced under the same conditions as those in Figure 5. The lack of prevention of the photoreduction of DCIP by the HU radical produced in darkness indicates that the inactivation of the electron transport activity was caused by an interaction between HU radicals and some photogenerated species of PS II component(s).

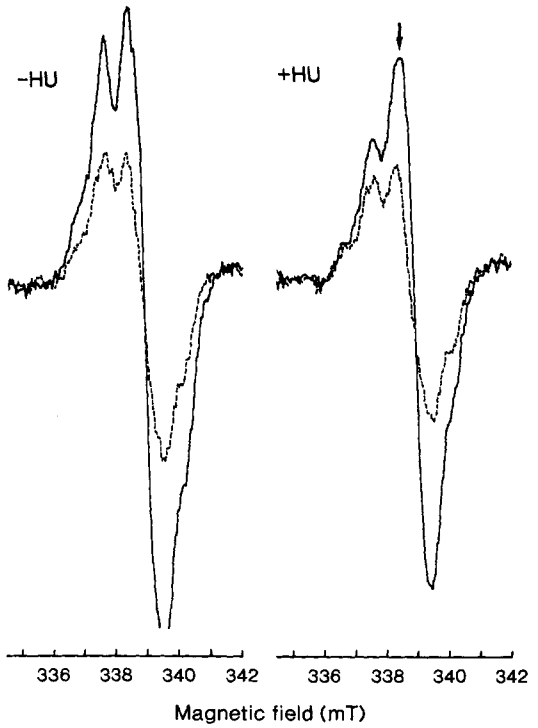

FIGURE 8: Changes in levels of $Y_Z^\bullet$ and $Y_D^\bullet$ upon treatment of Tris-treated PS II membranes with HU. Tris-treated PS II membranes were preincubated with or without 0.5 mM HU under illumination as described in the legend to Figure 6. After preincubation, the samples were diluted with buffer A, centrifuged at 35000*g* for 10 min, and suspended at 2 mg of Chl mL^{-1} with buffer A. The ESR spectrum was recorded in darkness (dashed line) and under illumination at 250 W m^{-2} (solid line) as described in Materials and Methods. Instrument settings were the same as those in the legend to Figure 2. The arrow indicates 338.4 mT.

Photogeneration of $Y_Z^\bullet$ Is Suppressed by Hydroxyurea Radicals. The photoproduced tyrosine radicals, $Y_Z^\bullet$ and $Y_D^\bullet$, in the D1 and D2 polypeptides, respectively, are possible sites of interaction with photoproduced HU radicals. To examine this possibility, effects of HU on the photogeneration of tyrosine radicals in Tris-treated PS II membranes were monitored. After incubation of Tris-treated PS II membranes with HU under illumination, the ESR signal from $Y_Z^\bullet$ was considerably reduced but that from $Y_D^\bullet$ was affected to a lesser extent. At the same time, the ESR signal at 338.4 mT was increased (Figure 8). This signal has been assigned to a cation radical of chlorophyll ($Chl^{\bullet+}$; Malkin & Bearden, 1973; dePaula et al., 1985), in the vicinity of the PS II reaction center, that is generated by $P680^+$ (Thompson & Brudvig, 1988). The yield of $Y_Z^\bullet$ upon illumination decreased by up to about 60% in the presence of 0.1 mM HU, but no further decrease was observed upon further increase in the concentration of HU (Figure 9). The profile of the suppression of the yield of $Y_Z^\bullet$ with changes in the concentration of HU was very similar to that of the photoreduction of DCIP (Figure 6). Thus, it is well possible that the photogenerated HU radicals modify $Y_Z^\bullet$ rather than $Y_D^\bullet$, with resultant inhibition of the oxidation of Y_Z by $P680^+$ and enhancement of the production of $Chl^{\bullet+}$.

DISCUSSION

Univalent Photooxidation of Hydroxyurea to Its Aminoxy Radicals by Water-Oxidizing Enzyme. NH_2OH and NH_2-NH_2 have been shown to interact with WOE by two distinct mechanisms: first, they interact with the Mn cluster of WOE and release Mn^{2+} to inactivate the enzyme in darkness

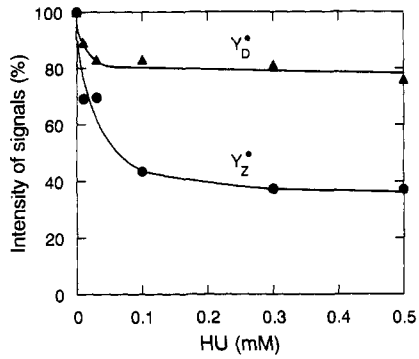

FIGURE 9: Dependence of the intensity of signals from $Y_Z^•$ and $Y_D^•$ on the concentration of hydroxyurea during preincubation. The preincubation conditions were the same as those described in the legend to Figure 6. After the preincubation, the samples were washed as described in the legend to Figure 8. ESR spectra were recorded as described in Materials and Methods. The samples (2 mg of Chl m^{-1}) were illuminated at 250 W m^{-2} for 1 min and incubated in darkness at room temperature. The logarithm of the height of the peak at 337.5 mT of the signal from the tyrosine radicals was plotted against the duration of dark incubation after illumination. The height of the peak upon illumination was regarded as the '0 min' value. It was assumed that the decaying component observed during the dark incubation period from 2 to 12 min after termination of illumination was $Y_D^•$. Then the decay phase in darkness was extrapolated linearly to the *y*-axis. The value on the *y*-axis was taken as the intensity of the signal from $Y_D^•$. The intensity of the signal from $Y_Z^•$ was calculated by subtracting the value for $Y_D^•$ from the '0 min' value on the *y*-axis.

(Cheniae & Martin, 1971; Yocum et al., 1981), and, second, they are photooxidized at the putative H_2O binding site as H_2O analogs (Radmer & Ollinger, 1983). HU also showed the above two effects on WOE. A slow inactivation by HU in darkness was accompanied by a release of manganese. HU was photooxidized in the oxygenic PS II membranes, and the primary product of HU oxidation at WOE was identified to be the aminoxy radical of HU by its ESR spectrum (Figures 2 and 3). This is the first report to directly demonstrate the generation of radical species by the univalent oxidation at the WOE, although the radical production has been assumed for NH_2OH (Radmer & Ollinger, 1982) and acetone hydrazone (Vass et al., 1990). By contrast to the univalent photooxidation of these compounds, the photooxidation of H_2O_2, another H_2O analog, at WOE is exclusively divalent (Mano et al., 1987).

Inactivation of PS II Reaction Center by Hydroxyurea Radicals Photoproduced at WOE. The oxygen-evolving activity of PS II membranes decreased in the presence of HU under the conditions where PS II turned over and HU radicals were photoproduced at the WOE (Figures 1–3). The inhibition of the oxygen evolution was caused mainly by the inhibition of the electron transport between Y_Z and Q_B (Figure 1B). The photoproduced HU radicals at the WOE decayed with first-order kinetics in darkness, an indication that little spontaneous dismutation or recombination of HU radicals occurred and that they interacted preferentially with sites in PS II (Figure 4). These results suggest that the photoproduced HU radicals at the WOE did not attack the WOE but the reaction center of PS II. The assessed distance between the Mn cluster and Y_Z is about 7 Å (Svensson et al., 1991), which allows an interaction of the HU radicals photoproduced at the WOE with Y_Z. As discussed below, HU radicals photoproduced by Tris-treated PS II membranes most probably interacted with $Y_Z^•$ to consequently inhibit the photoreduction of DCIP. Therefore, it is suggested that HU radicals photoproduced at the WOE also interacted with $Y_Z^•$ to consequently inhibit the oxidation of Y_Z by $P680^+$.

Photooxidation of Hydroxyurea to Its Aminoxy Radicals by Tris-Treated PS II Membranes. HU was also photooxidized to its aminoxy radicals by Tris-treated PS II membranes (Figure 2). The radical photoproduction by one-electron oxidation has been observed for *N*,*N*-dimethylhydroxylamine in Tris-treated PS II membranes (Beck & Brudvig, 1987) as well as for HU. A candidate for the oxidant of HU is $Y_Z^•$. Since $Y_Z^•$ has been shown to be exposed to hydrophilic environments (Svensson et al., 1991), HU is able to interact with $Y_Z^•$. By contrast, $Y_D^•$ is located in a hydrophobic environment (Babcock et al., 1989; Svensson et al., 1991) and is unlikely to be a site of the oxidation of HU. In fact, $Y_D^•$ in Tris-treated PS II membranes was only very slowly reduced by HU in darkness, with a half-time of 30–40 min (data not shown).

Interaction of Hydroxyurea Radicals with $Y_Z^•$ in Tris-Treated PS II Membranes. In Tris-treated PS II membranes, the photoproduced HU radicals interact with photoproduced species of PS II component(s), as indicated from the absence of inactivation of the sample in darkness by HU radicals produced by horseradish peroxidase and hydrogen peroxide. The photoproduction of $Y_Z^•$ was suppressed and production of $Chl^{•+}$ was enhanced by HU radicals under the conditions where PS II turned over (Figures 8 and 9). The *in situ*-photoproduced HU radical is, therefore, proposed to interact with photogenerated $Y_Z^•$. The suppressions of photoreduction of DCIP and of the photogeneration of $Y_Z^•$ were similarly dependent on the concentration of HU (Figures 6 and 9), suggesting that the modification leading to loss of $Y_Z^•$ by HU radicals was responsible for the decrease in DCIP-photoreducing activity. An alternative site of interaction with HU radicals might be $Y_D^•$, but it does not seem to be a major site of the interaction because the photoproduction of $Y_D^•$ was only slightly affected by HU radicals (Figure 9). This small suppression of $Y_D^•$ is probably a secondary effect caused by the modification of Y_Z by HU. $Y_Z^•$ is, therefore, most likely the site of interaction with HU radicals.

Our results suggest that the photoinactivation of Tris-treated PS II membranes by HU is caused by a radical–radical interaction, namely, the reaction of HU radical with the radical of Y_Z. Ribonucleotide reductase is also inactivated by HU, but HU inactivates the enzyme only by reduction of the functional tyrosine radical (Karlsson et al., 1992; Lassmann et al., 1992) unlike PS II. Inactivation of PS II by the interaction of $Y_Z^•$ with other radicals has been also reported by Chen et al. (1992b), who have provided evidence that $Y_Z^•$ interacts with superoxide anion radicals, which are photoproduced by univalent reduction of dioxygen on the acceptor side of PS II in hydroxylamine-treated PS II membranes.

Electron transport through Y_Z was partially alive even after photoinactivation of Tris-treated PS II membranes with HU (Figure 7). This suggests the existence of a population of $Y_Z^•$ that does not further oxidize HU nor react with HU radicals. Heterogeneity of PS II has been demonstrated by Melis (1990) with regard to the size of the antenna (PS IIα, with a larger antenna, and PS IIβ, with a smaller one). However, it is not clear whether the heterogeneity of $Y_Z^•$ in terms of reactivity with HU radicals is in any way correlated with that of the size of the antenna. Alternatively, it is possible that the HU-modified Y_Z is redox active, as the report that the iodinated Y_D can be oxidized by $P680^+$ (Takahashi & Styring, 1987).

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