# Induction of Luminol Chemiluminescence by the Manganese Cluster of the Photosystem II Water-Oxidizing Complex in the $S_0$, $S_1$, $S_2$, and $S_3$ States†

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*Received June 30, 1995; Revised Manuscript Received December 7, 1995*⊗

ABSTRACT: Luminol chemiluminescence (CL) ($\lambda_{max}$ = 425 nm) induced by the manganese cluster of photosystem II (PSII) ($t_{max}$ = 1−5 min) along with CL induced by mono-, di-, or tetranuclear manganese coordination complexes ($t_{max}$ = 2−40 s) is observed when either 200 mM sodium phosphate or 1 M Tris-HCl + 200 $\mu$M EDTA are present in the reaction medium (pH 8.5) containing peroxidase. This light emission is not observed when Tris is used in the reaction medium without EDTA. The yield of a given CL without peroxidase is 5%−20% of that with peroxidase. The peroxidase-dependent CL is inhibited by catalase ($I_{50}$ = 0.14−0.17 $\mu$M), while the peroxidase-independent CL is not inhibited by 100 mM ethanol, 1 mM $NaN_3$, 20 $\mu$M Cyt *c*, or 0.6 $\mu$M catalase. The CL induced by the Mn cluster of the water-oxidizing complex (WOC) in the $S_3$ or $S_2$ state exceeds that of lower S states by 15−20-fold. The magnitude of CL induced by Mn complexes is dependent on the ligand type of the complex. The ligand types of the Mn complexes and the WOC in different S states are ranked according to the magnitude of the induced CL: 1,10-phenanthroline > 2,2′-bipyridine > WOC ($S_3$ or $S_2$) > hydrotris(pirazolyl) borate > WOC ($S_{0-2}$ or $S_0$, $S_1$) > 1,4,7-triazacyclononane. It is concluded that CL is caused by $H_2O_2$ formed as a result of the oxidation of luminol by triplet molecular oxygen. The Mn cluster of WOC and manganese coordination complexes, acting as catalysts of this reaction, show oxidase activity. Upon $S_2-S_3$ or $S_1-S_2$ transition, changes occur in the ligand environment of the Mn cluster of WOC influencing the induction of luminol CL.

Photosystem II (PSII),[1] catalyzing the light-driven oxidation of water and the reduction of plastoquinone, is a pigment−protein complex that is anisotropically incorporated into the thylakoid membrane [for recent reviews see Hansson and Widrzynski (1991) and Debus (1992)]. The antenna complex of PSII absorbs light and transfers the excitation energy to the reaction center (RC) and ultimately to a specialized monomer or dimer of chlorophyll *a* molecules known as P680 [for recent reviews see Renger (1992)]. In the RC there occurs an energy conversion from the excited singlet state of P680 to free energy of separated charges forming $P680^+$ and $Pheo^-$ (Klimov & Krasnovsky, 1981). The rapid oxidation of $Pheo^-$ by the plastoquinone molecule $Q_A$ and the reduction of $P680^+$ by a redox-active tyrosine residue $Y_z$ of the protein D1 prevent charge recombination (Hoganson & Babcock, 1988). Charge recombination between $Q_A^-$ and $Y_z^+$ is prevented by the oxidation of $Q_A^-$ by a second plastoquinone molecule $Q_B$ and the reduction of $Y_z^+$ by the water-oxidizing complex (WOC) (Crofts & Wraight, 1983). Four consecutive charge separations create an oxidation equivalent that is necessary to oxidize two water molecules to one molecule of molecular oxygen. The active site of WOC most likely includes four atoms of Mn (Cheniae & Martin, 1970). The WOC cycles through five intermediate states known as $S_i$ states ($i$ = 0−4) which reflect the oxidation states of Mn (Kok et al., 1970). $O_2$ evolution occurs during the $S_3-(S_4)-S_0$ transition. The $Ca^{2+}$ and $Cl^-$ ions are essential cofactors for the oxidation of water (Ghanotakis et al., 1984; Govindjee et al., 1983).

The structure and ligand environment of the manganese cluster of WOC in its different S states and the mechanism of water oxidation remain subjects of discussion due to the difficulty in directly measuring the oxidation and electron states of Mn in each S state. The basic methods for studying the given problem are EPR (Dismukes & Siderer, 1981; Hansson et al., 1987), X-ray (Yachandra et al., 1988; George et al., 1989), and optical absorption spectroscopy (Saygin & Witt, 1987) along with the interpretation of results based on the comparative structural analysis of synthetic manganese coordination complexes. Two EPR signals centered at $g$ = 2 (Dismukes & Siderer, 1981) and $g$ = 4.1 (Zimmermann & Rutherford, 1986) have been attributed to the Mn cluster in the paramagnetic $S_2$ state. The g = 2 signal presumably originates as a result of the magnetic coupling between the 2−4 atoms of Mn in the mixed-valence state, including Mn(III) and Mn(IV). The model of the PSII Mn cluster, put forth by Sauer et al. (1992) on the basis of EXAFS data, assumes a structure consisting of two di-$\mu_2$-oxo bridged Mn dimers which are connected with one mono-$\mu_2$-oxo and two

† This work was supported by the International Science Foundation (MTZ 000) and the Russian Fundamental Research Fund (93-04-20695).

⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1996.

[1] Abbreviations: Chl, chlorophyll; CL, chemiluminescence; Cyt *c*, cytochrome *c*; D1, a polypeptide of the photosystem II core; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; dinoseb, 2,4-dinitro-6-(2-butyl)phenol; DMSO, dimethyl sulfoxide; $\Delta F$, photoinduced changes of Chl fluorescence yield; $E°$, redox potential; EDTA, ethylenediaminetetraacetic acid; $F_0$, constant fluorescence; FWHM, full width at half-maximum; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PSII, photosystem II; P680, primary electron donor of photosystem II; $Q_A$ and $Q_B$, primary and secondary plastoquinone acceptors of photosystem II; RC, reaction center of water-oxidizing complex; $S_i$, redox state *i* of the water oxidase; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; WOC, water-oxidizing complex; $Y_z$, redox-active tyrosine of polypeptide D1 that donates an electron to $P680^+$.

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carboxylato bridges. It is assumed that the ligands of Mn ions in PSII may be carboxylate residues (Preston & Seibert, 1991) and imidazole groups of histidine residues (DeRose et al., 1991).

Earlier, Ananyev and Klimov (1989) observed a slow CL of a luminol−peroxidase system (signal B) induced by illuminated PSII particles. The CL was interpreted to be a result of the interaction of the CL system with the oxidized Mn of PSII. The amplitude of signal B reaches a maximum 60−90 s after injection the illuminated PSII particles into the luminol−peroxidase system. An addition of 0.08 $\mu$M catalase to the sample before measuring CL does not inhibit the light emission. The creation of anaerobic conditions in the suspension of PSII particles before illumination hardly affects the signal. In dark conditions lacking the luminol−peroxidase system signal B fades out to 10% of its original magnitude within 11−12 min. Signal B disappears when Mn is completely removed from the PSII particles and is not restored when sodium ascorbate is added as an electron donor. Inhibitors of electron transfer in PSII such as DCMU, *o*-phenanthroline, and dinoseb eliminate signal B.

In this work the mechanism of this slow CL is investigated with the aim of further using the luminol−peroxidase system as a new approach to studying the structural and functional organization of the PSII WOC. The intensity of CL induced by the Mn cluster of WOC is shown to differ according to the S state and further depends on the ligand environment.

## MATERIALS AND METHODS

Studies were performed on $O_2$-evolving [200−300 $\mu$mol of $O_2$ (mg of Chl)$^{-1}$ h$^{-1}$] PSII particles isolated from spinach using Triton X-100 (Berthold et al., 1981). To remove the weak-binding divalent cations, the samples were washed twice before the beginning of the measurements in a medium containing 40 mM Mes-NaOH, pH 6.5, 35 mM NaCl, 300 mM sucrose, and 0.5 mM EDTA.

Luminol CL with $\lambda_{max}$ = 425 nm was measured as described earlier (Ananyev & Klimov, 1988). Before the measurement, 50 $\mu$L of PSII particles (0.5−1 mg of Chl/mL) held in a clear plastic conical pipette ($d$ = 1.5−3.5 mm, $h$ = 20 mm) was illuminated with a series of light flashes and then injected after 10 s into a reaction medium containing 200 mM sodium phosphate buffer, pH 8.0−8.5, 60−200 $\mu$M luminol, and 0.5−1 mM horseradish peroxidase. The flash energy was 7 J, the FWHM was 1 $\mu$s, and the frequency was 1 Hz.

The rate of $O_2$ evolution by PSII particles (10−20 $\mu$g of Chl/mL) was measured in a 1 mL chamber using a Clark-type horizontal electrode. The sample was illuminated with red light (I = 100 W m$^{-2}$) passed through a 20 mm layer of 2% $CuSO_4$. The flash-induced $O_2$ yield was measured with an analog electrode covered by a Teflon membrane in a 20 $\mu$L chamber with a height of 0.2 mm. The assay medium contained 40 mM Mes-NaOH, pH 6.5, 35 mM NaCl, and 300 mM sucrose, with 0.4 mM $K_3Fe(CN)_6$ and 0.4 mM DCBQ as electron acceptors. The energy of the flash was 9 J, the FWHM was 1 $\mu$s, and the frequency was 1 Hz.

Photoinduced changes in the PSII chlorophyll fluorescence yield ($\Delta F$) was measured on a phosphoroscopic setup as described earlier (Klimov et al., 1985). The content of Mn in the PSII particles was determined by means of an atomic absorption spectrophotometer (Perkin-Elmer AAS-503).

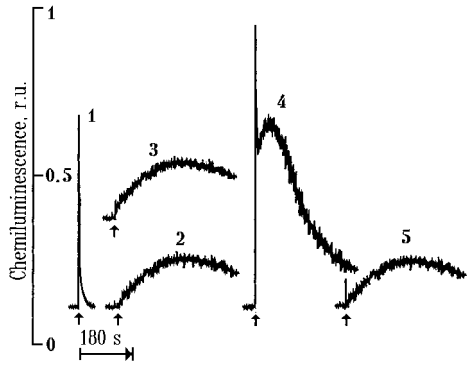


FIGURE 1: CL of the luminol−peroxidase system induced by 10 nM $H_2O_2$ (1); 30 min dark-adapted PSII particles (2); illuminated with one light flash (3); illuminated with five light flashes (4); illuminated with five light flashes in the presence of 20 $\mu$M DCMU (5). Arrows indicate the moment the sample was injected into the reaction medium.

Manganese from PSII particles was removed (>90%) by incubating the sample (0.25 mg of Chl/mL) in medium containing 20 mM Mes-NaOH, pH 6.5, and 20 mM TEMED for 5 min in the dark at 4 °C, as described earlier (Ananyev et al., 1992).

The influence of the reaction medium, used for measuring CL, on the activity of PSII particles was studied by incubating 10-flash illuminated PSII particles in medium containing 50−200 mM phosphate buffer, pH 8.0−8.5, 15 mM sucrose, 1.7 mM NaCl, 0.5 $\mu$M peroxidase, 120 $\mu$M luminol, at 4 °C in the dark with a chlorophyll concentration of 50 $\mu$g/mL. After incubation the PSII particles were pelleted and resuspended in medium containing 40 mM Mes-NaOH, pH 6.5, 35 mM NaCl, and 300 mM sucrose.

Synthetic Mn complexes were kindly donated by Professor G. C. Dismukes: $((phen)_2Mn^{III}(\mu\text{-}O)_2Mn^{IV}(phen)_2)^{3+}$ (complex 1), where phen = 1,10-phenanthroline; $((bipy)_2Mn^{III}(\mu\text{-}O)_2Mn^{IV}(bipy)_2)^{3+}$ (complex 2), where bipy = 2,2′-bipyridine; $(Mn^{III}(phen)_3)^{3+}$ (complex 3); $(HBPz_3)Mn^{III}(\mu\text{-}O)(\mu\text{-}O_2CR)_2Mn^{III}(HBPz_3)$ (complex 4), where R = $CH_3$ and $HBPz_3$ = hydrotris(pirazolyl)borate; $((TACN)Mn^{III}(\mu\text{-}O)_2(\mu\text{-}O_2CR)Mn^{IV}(TACN))^{2+}$ (complex 5), where TACN = 1,4,7-triazacyclononane and R = $CH_3$; and $((TACN)_4Mn_4{}^{IV}O_6)^{4+}$ (complex 6) [for details see Tang et al., 1993)]. Complexes were dissolved in aprotic DMSO to a concentration of 1mM. The dissolved complexes were kept no more than 6 h. Immediately before measurement the complexes were diluted to a concentration of 10 $\mu$M in either DMSO or $H_2O$.

Calculation of the $S_i$ population was performed according to the Kok model (Kok et al., 1970): $S_i = (1 - \alpha - \beta)S_{i-1} + \alpha S_i + \beta S_{i-2}$, where $\alpha$ represents misses and $\beta$ represents double hits.

## RESULTS

*Luminol CL Induced by PSII Particles and Mn Compounds.* PSII particles, unilluminated or illuminated with one light flash and dark-adapted for 30 min at 4 °C, injected into a luminol−peroxidase system induce a slowly increasing CL (called signal B′), with a maximum intensity after 3−5 min of mixing (Figure 1). PSII particles illuminated with five light flashes induce CL, consisting of fast (signal A) and slow (signal B) components which reach their maxima in 0.2−0.3 and 60−90 s, respectively. Signal A caused by $H_2O_2$ and forming as a result of the reduction of $O_2$ by

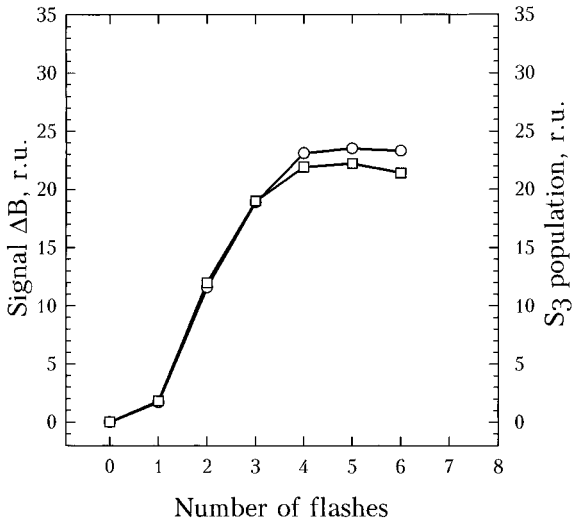


FIGURE 2: Signal ΔB (○) and the calculated $S_3$ population of WOC ($S_0 = 25\%$, $S_1 = 75\%$, $\alpha = 0.54$, $\beta = 0.03$, with decay coefficients of 0.15 and 0.2 for $S_2-S_1$ and $S_3-S_2$ transitions, respectively) (□) as a function of the number of light flashes.

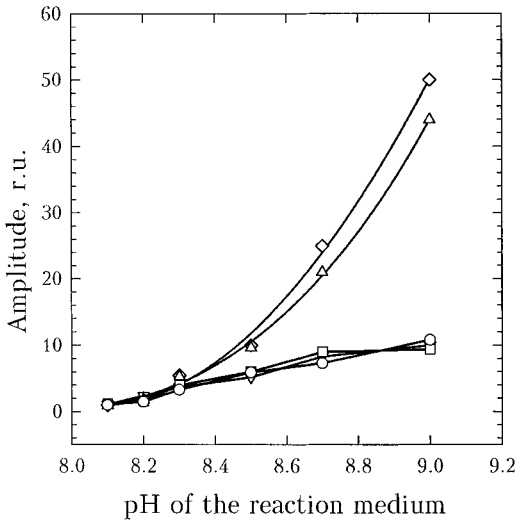


FIGURE 3: Dependence of the intensity of the CL of the luminol−peroxidase system, induced by 1 $\mu$M $H_2O_2$ (□), 40 $\mu$M $MnCl_2$ (▽), or 10 $\mu$M complex 4 (○); signal B′ (△) and ΔB (◇) on pH of the reaction medium.

components of the acceptor side of PSII (Ananyev & Klimov, 1988) is not further considered in this work. The magnitude of signal B exceeds that of signal B′ by a factor of 2−6. Only signal B′ is induced when 20 $\mu$M DCMU is added to the PS II particles prior to illumination. Therefore, the difference between signals B and B′ (ΔB) is light-induced and dependent on electron transfer in PSII. It is necessary to note that we have named signal ΔB what was earlier (Ananyev & Klimov, 1989; Klimov et al., 1993) considered signal B. This is explained by the fact that in earlier works signal B′ did not register, probably due to the relatively low pH of the reaction medium (pH 8.0).

The magnitude of signal ΔB depends on the number of light flashes as shown in Figure 2. The Kok model was used to determine with which S state signal ΔB is associated. The measurement of CL is made 10 s after the illumination of the sample. Therefore, the calculations reflect the expected 10%−40% transition from $S_2$ and $S_3$ to lower S states that takes place during that time period (Messinger & Renger, 1994; Joliot et al., 1971). Figure 2 shows the distribution of the calculated $S_3$ population in conditions where $\alpha = 0.54$, $\beta = 0.03$, $S_0 = 25\%$, and $S_1 = 75\%$ (the initial distribution of the S states in the dark) and the decay coefficients are 0.15 and 0.2 for $S_2-S_1$ and $S_3-S_2$ transitions, respectively.

Signals B and B′ are eliminated upon removal of Mn from PSII particles by 20 $\mu$M TEMED and are not restored following addition of 40 $\mu$M sodium ascorbate, an artificial electron donor for PSII (data not shown). Therefore these signals are connected with the Mn of the WOC.

Earlier it was suggested that $MnCl_2$ induces luminol CL ($t_{max}$ = 1 min) similar to signal B (Ananyev & Klimov, 1989). In the present work, synthetic manganese complexes (see Materials and Methods) along with $MnCl_2$ are used. These induce CL, the intensity of which reaches a maximum 2−40 s after the beginning of the measurement and continues for 10−40 min. Their induction of luminol CL proceeds in the absence of $H_2O_2$ additions to the reaction medium. As shown in Figure 3, the intensity of CL, induced by either Mn compounds or 1 $\mu$M $H_2O_2$, increases 10 times when the pH of the reaction medium increases from pH 8.1 to 9.0.

Table 1: Protein Concentration in the Reaction Medium at pH 8.5 Which Inhibits Luminol CL Induced by Either $H_2O_2$, Mn Compounds, or PSII Particles by 50%

| initiators of luminol CL | $I_{50}$, $\mu$M | | | | |
|---|---|---|---|---|---|
| | catalase | Cyt *c* | hemoglobin | peroxidase | albumin |
| 1 $\mu$M $H_2O_2$ | 0.16 | 16.1 | 5.1 | 9.2 | *a* |
| 40 $\mu$M $MnCl_2$ | 0.15 | 15.8 | 4.8 | 10.1 | |
| 10 $\mu$M complex 4 | 0.17 | 16.8 | 5.3 | 9.4 | |
| PSII particles | | | | | |
| signal ΔB | 0.14 | 17.5 | 4.8 | 9.8 | |
| signal B′ | 0.15 | 17.1 | 4.6 | 10.5 | |

[a] Inhibition was not observed.

This is explained by the fact that luminol CL proceeds only in an alkaline medium with a maximum at pH 10−11 (Bostik & Hercules, 1975). At the same time, the intensities of signals ΔB and B′ increase about 50 times with pH increases from 8.1 to 9.0 (Figure 3, curves △ and ◇).

*Participation of $O_2$ and Its Active Forms in the Induction of Luminol CL.* Addition of 100 mM ethanol (a trap for •OH) or 1 mM $NaN_3$ (a quencher of singlet oxygen) to the reaction medium does not affect the CL (data not shown).

Cyt *c* and catalase added to the reaction medium inhibit luminol CL induced by both PSII particles (signals B and B′) and Mn compounds (Table 1). However, the effectiveness of the inhibition of CL by catalase is approximately 2 orders of magnitude greater than that of Cyt *c*. Similarly, heme-containing proteins (differing from albumin) such as hemoglobin and peroxidase inhibit CL at concentrations close to that of Cyt *c*. All heme-containing proteins inhibit CL induced by 1 $\mu$M $H_2O_2$ at concentrations similar to those which inhibit CL induced by PSII particles and Mn compounds. Addition of 0.06 $\mu$M catalase directly to the PSII particles or Mn compounds hardly affects CL. However, if the same concentration of catalase is added to 1 $\mu$M $H_2O_2$, then CL is not observed (data not shown).

The participation of peroxidase is a necessary condition for the oxidation of luminol by $H_2O_2$ (Tokmakov, 1993). However, signals B and B′ along with CL induced by Mn complexes are observed in the absence of peroxidase, but the intensity of the light emission is 5%−20% of that

Table 2: Dependence of Luminol CL Expressed in Molecules of $H_2O_2$ (see Text) per Atom of Mn (Mn Compounds) or per RC (PSII Particles Unilluminated and Illuminated by 10 Light Flashes) on the Type of Buffer in the Reaction Medium in the Presence or Absence of 200 $\mu$M EDTA[a]

| initiators of luminol CL | type of buffer in reaction medium | | | | | |
|---|---|---|---|---|---|---|
| | 200 mM phosphate | 200 mM phosphate + 200 $\mu$M EDTA | 1 M Tris-HCl | 1 M Tris-HCl + 200 $\mu$M EDTA | 50 mM Hepes | 50 mM Hepes + 200 $\mu$M EDTA |
| 10 $\mu$M complex 1 | 0.35 (0.5) | 0.15 (0.4) | 0 (0) | 0.02 (0.06) | 0 (0) | 0.01 (0.07) |
| 10 $\mu$M complex 2 | 0.32 (0.8) | 0.02 (0.15) | 0 (0) | 0.01 (0.01) | 0.01 (0.01) | 0.01 (0.01) |
| 10 $\mu$M complex 3 | 1.5 (1.4) | 0.5 (0.4) | 0 (0) | 0.1 (0.01) | 0.14 (0) | 0.08 (0.02) |
| 10 $\mu$M complex 4 | 0.2 (0.7) | 0.02 (0.15) | 0 (0) | 0.01 (0.03) | 0 (0) | 0 (0.01) |
| 10 $\mu$M complex 5 | 0.02 (0.2) | 0 (0.01) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 10 $\mu$M complex 6 | 0 (0.01) | 0 (0.03) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 40 $\mu$M $MnCl_2$ | 0.06 | 0 | 0 | 0 | 0 | 0 |
| PSII Particles | | | | | | |
| (signal $\Delta$B) | 0.15 (0.6) | 0.09 (0.35) | 0[b] | 0.05(0.2) | 0[b] | 0[b] |
| (signal B′) | 0.04 | 0.02 | 0[b] | 0.01 | 0[b] | 0[b] |

[a] For Mn complexes, values in parentheses indicate dilution prior to measurement in DMSO as opposed to $H_2O$; for PSII particles, values in parentheses indicate the magnitude is calculated per WOC in the $S_3$ state. [b] It is possible that this is not a zero value; due to the length of the duration of the light emission and the small amplitude of the signal, it may not register.

registered in the presence of peroxidase. In this case CL is not inhibited by 0.6 $\mu$M catalase, 20 $\mu$M Cyt *c*, 100 mM ethanol, and 1 mM $NaN_3$ (data not shown).

*Dependence of the Intensity of Luminol CL on the Type of Buffer in the Reaction Medium.* As shown above, CL's maximum yield is strongly dependent on peroxidase. Quantitative evaluation (in terms of molecules of $H_2O_2$ per RC or Mn atom) of a given light emission is therefore made possible, i.e., the area of a CL signal is relative to the area of the CL signal induced by the control 1 $\mu$M $H_2O_2$ measured in a corresponding reaction medium. As shown in Table 2 the intensity of luminol CL induced by PSII particles (signals B′ and $\Delta$B) and Mn compounds is dependent on the presence of either 200 mM phosphate, 1 M Tris, or 50 mM Hepes buffer, pH 8.5, with or without 200 $\mu$M EDTA in the reaction medium. The intensity of signal $\Delta$B is also expressed in terms of molecules of $H_2O_2$ per WOC in the $S_3$ state. In these experiments, after illumination with 10 light flashes the distribution of the S states is approximately equally divided. Therefore, the concentration of the WOC in the $S_3$ state was about 25% of the concentration of the RC. Signal B was calculated per RC for dark-adapted particles when $S_0 + S_1 = 100\%$. The intensities of signals B′ and $\Delta$B differ according to the reaction medium. As shown in Figure 4 the applied mediums show differences according to their level of modification of the donor side of PSII. This can be determined from the observed decrease in the rate and magnitude of $\Delta F$, which is related to the photoreduction of $Q_A$, measured in these mediums. However, there is no correlation between the intensity of slow luminol CL induced by PSII particles and the level of damage to the donor side of PSII (compare Figure 4 with Table 2).

The luminol CL induced by 40 $\mu$M $MnCl_2$ is observed only when the reaction medium consists of a phosphate buffer (Table 2). The presence of 200 $\mu$M EDTA which chelates Mn(II) inhibits this light emission. If $MnCl_2$ is incubated in 200 mM phosphate buffer at pH 8.5 for 5 min before the beginning of the measurement, then the time to the CL maximum shortens from 60 to 45 s while the amplitude of the light emission increases insignificantly (data not shown). The intensity of CL induced by Mn complexes is maximized in a phosphate buffer (Table 2). An addition of EDTA into the reaction medium with phosphate buffer partly inhibits this light emission (except complex 6). In

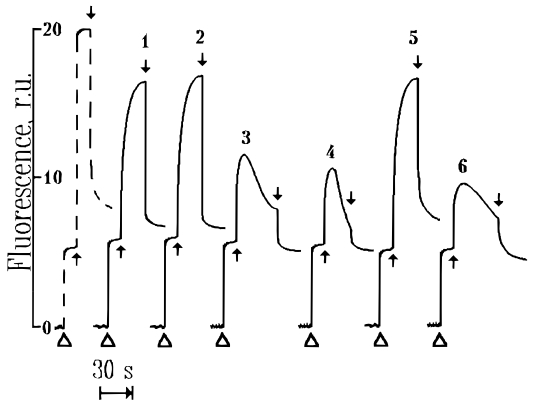


FIGURE 4: Kinetics of photoinduced changes in the PSII chlorophyll fluorescence yield ($\Delta F$), measured in an assay medium containing 200 $\mu$M luminol, 0.5 $\mu$M peroxidase and different additions: 200 mM phosphate, pH 8.5 (1); 200 mM phosphate, pH 8.5, and 200 $\mu$M EDTA (2); 1 M Tris-HCl, pH 8.5 (3); 1 M Tris-HCl, pH 8.5, and 200 $\mu$M EDTA (4); 50 mM Hepes-NaOH, pH 8.5 (5); 50 mM Hepes-NaOH, pH 8.5, and 200 $\mu$M EDTA (6). The dashed line shows the kinetics of $\Delta F$, measured in medium containing 35 mM NaCI and 40 mM Mes-NaOH, pH 6.5. A triangle ($\triangle$) indicates the moment of switching on the measuring light ($\lambda_{max} = 480$ nm, 0.1 W $m^{-2}$). Arrows indicate the switching on ($\uparrow$) and off ($\downarrow$) of the actinic light ($\lambda > 600$ nm, 100 W $m^{-2}$). The measuring of $\Delta F$ took place after a 2 min incubation of the particles in the assay medium.

cases when PSII particles and complexes 1−4 act as initiators of CL, light emission is not observed in the presence of Tris buffer in the reaction medium, while a following addition of EDTA brings about the light emission. No correlation is found between the intensity of CL induced by Mn complexes and such features as the nuclearity of the complexes, the bridge type between their Mn atoms, and the valency of Mn. CL induced by the complexes which were diluted before measurement in $H_2O$ is less intense than that induced by complexes diluted in DMSO (except complex 3).

*Influence of the CL Reaction Medium on the Activity of PSII Particles.* Interaction between the reaction medium, which is used for the registration of $H_2O_2$, and PSII particles causes a decrease in the $O_2$-evolving activity of the PSII particles (Figure 5). Furthermore, the $O_2$ evolution during continuous illumination is inhibited significantly faster than $O_2$ yield on the third light flash. Mn content in the particles and the $\Delta F/F_0$ ratio decrease by 15% and 30%, respectively,

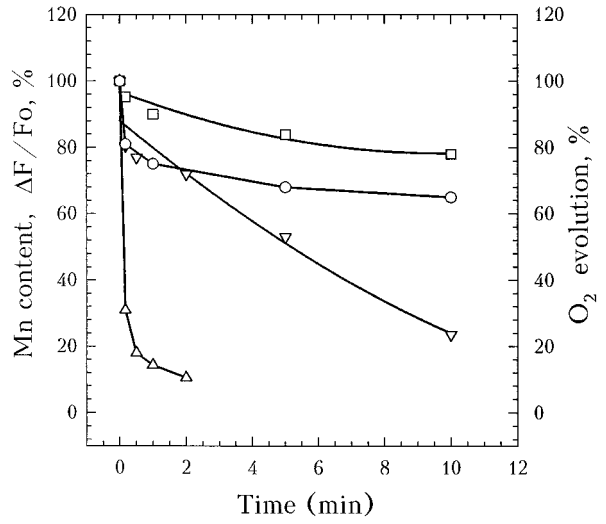


FIGURE 5: Dependence of the rate of $O_2$ evolution by PSII particles in continuous illumination (△), the $O_2$ yield on the third saturating light flash (▽), the $\Delta F/F_0$ ratio (○), and the amount of Mn in the PSII particles (100% corresponds to 4 Mn per 250 molecules of Chl) (□) on the incubation time in CL medium. The amount of Mn and $O_2$ evolution were measured after incubating the particles in the CL medium containing 50 mM phosphate buffer, pH 8.0. ΔF was measured after incubation in medium containing 200 mM phosphate buffer, pH 8.5.

after 5 min incubation (Figure 5). Thus when PSII particles are incubated in the reaction medium a modification of the Mn cluster in the WOC occurs. At the same time, complete destruction is not observed.

*Role of the CL System in $H_2O_2$ Formation.* In order to ascertain which component of the reaction medium takes part in the $H_2O_2$ formation inducing slow CL, illuminated PSII particles or complex 4 are initially incubated in 200 mM phosphate buffer, pH 8.5, in both the presence and absence of luminol or peroxidase. Thus, the decrease in the time to the light emission maximum to 0.2−0.3 s and at the same time the increase in its amplitude may testify to the formation of $H_2O_2$ during incubation. As shown in Figure 6, the kinetics of CL induced by complex 4 initially incubated in phosphate buffer do not differ from those induced by complex 4 diluted in water before the measurement. The kinetics and amplitude of signal B after incubation of PSII particles in phosphate change insignificantly. As a side note, signal A decreases by about 5-fold, most likely due to the catalase activity of PSII (Velthuys & Kok, 1978) and/or the interaction of $H_2O_2$ with components of the photosynthetic membrane. Addition of 10 $\mu$M peroxidase does not bring about additional effects; $H_2O_2$, causing signal A, in this case is completely decomposed by peroxidase. The addition of 2.4 mM luminol inhibits signal B by about 70% and significantly changes the CL kinetics induced by complex 4 (shortening the time to the maximum from 5 to 0.3 s and increasing the amplitude 4-fold), while the total light emission (area of the CL signal) insignificantly changes.

## DISCUSSION

These results show that the slow CL induced by unilluminated PSII particles and the CL induced by illuminated PSII particles are similar in terms of their mechanism of formation and only differ in the fact that signal ΔB is photodependent whereas signal B′ is not. Signal B′ is associated with the $S_0$ and $S_1$ states and probably also with

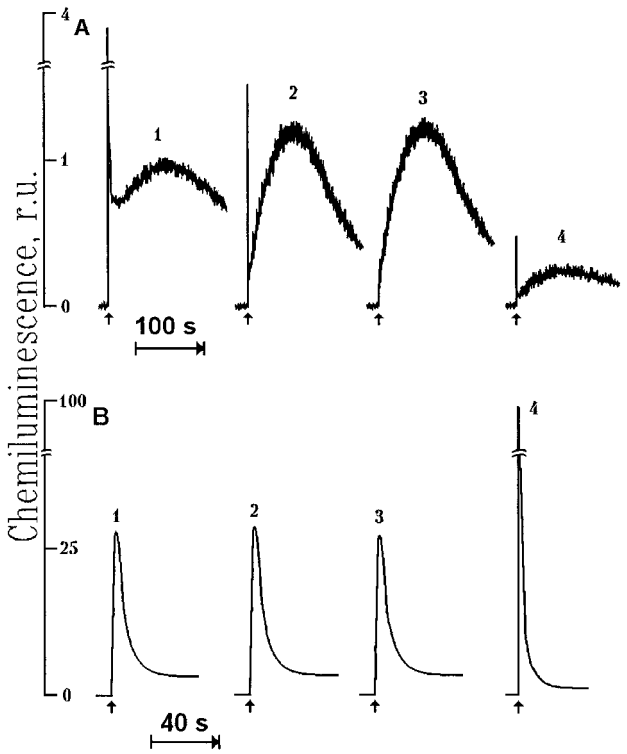


FIGURE 6: CL of the luminol−peroxidase system, induced by PSII particles illuminated by five light flashes (A), and complex 4 diluted in $H_2O$ before measuring (B). The control measurement is not incubated (1). PSII particles or complex 4 are preliminary incubated for 5 or 2 min, respectively, in 200 mM phosphate buffer, pH 8.5 (2), in 200 mM phosphate buffer (pH 8.5) + 10 $\mu$M peroxidase (3), in 200 mM phosphate buffer (pH 8.5) + 2.4 mM luminol (4).

the $S_2$ state. It is hardly affected by changes in the $S_0/S_1$ ratio and the appearance of the $S_2$ state in response to the first light flash (the $S_3$ population, occurring as a result of double hits, is insignificant at the time of the first light flash and therefore can be ignored). Signal ΔB, which is insignificant on the first light flash but abruptly increases on the second light flash, is likely to be associated with the $S_3$ state of WOC. This is confirmed by the fact that calculated changes in the $S_3$ population are very close to the experimental value for signal ΔB, using the above specified values for α and $\beta$. A relatively high value for α equal to 0.54 is most likely due to the high optical density of the sample. The date, however, do not provide an unambiguous proof for the origin of signal ΔB due to the lack of its clear oscillatory behavior. One cannot exclude the possibility of extremely fast decay of $S_2$ and $S_3$ to lower S states proceeding after injection of PSII particles into the reaction medium. In this case signal ΔB may come from the $S_2$ state formed as a result of the $S_3$ decay.

As known (Tokmakov, 1993), luminol CL in a protic medium may be brought about by triplet or, possibly, singlet $O_2$ along with its reduced forms: superoxide radicals, hydroxyl radicals, and $H_2O_2$. Present results demonstrate that the investigated CL is inhibited by catalase at lower concentrations than other heme-containing proteins and therefore imply that the CL is induced by $H_2O_2$. The inhibiting effect of Cyt *c* is of the same level as that of hemoglobin and peroxidase, probably connected with the fact that it contains heme iron which may have redox interaction with $H_2O_2$ (Wang, 1955). The fact that 0.06 $\mu$M catalase, an effective decomposer of $H_2O_2$, does not inhibit CL when

added to PSII particles and Mn compounds before they are injected into the reaction medium is explained by the formation of $H_2O_2$ (which causes the given light emission) during the processes occurring after the sample is injected into the reaction medium. However, the intensities of signals B′ and ΔB do not correlate with the level of damage to the donor side of PSII, as shown by the experiments where Tris and Hepes buffers are used in the place of phosphate buffer. At the same time, Tris, Hepes, EDTA and phosphate may interact with Mn(II) and Mn(III), changing its properties (Stadtman et al., 1990; Archibald & Fridovich, 1982; Frasch & Cheniae, 1980; Rickert et al., 1991). As with signals B′ and ΔB, changes in the intensity of CL induced by Mn compounds are also dependent on the type of buffer present in the reaction medium. In this way, signals B′ and ΔB are caused by $H_2O_2$, the formation of which is connected with a specific property of Mn. CL induced by Mn compounds and CL induced by PSII particles are therefore similar in nature.

How can $H_2O_2$ form with the participation of Mn? As explained earlier (Bostick & Hercules, 1975), a coordination complex having superoxide dismutase (SOD)-like activity forms upon interaction of Mn(II) with orthophosphate ions. This is connected with a decrease in the redox potential ($E°$) of the Mn(II)/Mn(III) pair upon the formation of the above mentioned complex. However, in these experiments preliminary incubation of $MnCl_2$ with phosphate only slightly changes the kinetics of CL, excluding the possibility of $H_2O_2$ formation upon oxidation of Mn(II) by molecular oxygen even with a decrease in the thermodynamic threshold for that reaction. This also demonstrates the participation of luminol or peroxidase in the formation of $H_2O_2$. Theoretically, the formation of $H_2O_2$ with the participation of the PSII Mn cluster and synthetic Mn complexes may be connected with the structural rearrangement of a complex upon the destruction of $\mu$-oxo bridges or hydrolysis. However, a preliminary incubation of PSII particles and complex 4 in a phosphate buffer hardly changes the kinetics of CL (i.e., a preliminary formation of $H_2O_2$ does not occur), eliminating the possibility of the above mentioned reaction. At the same time, the presence of luminol inhibits signal B, and in the case with complex 4 the increase in the amplitude of CL and shortening of the time to its maximum could demonstrate the participation of luminol in the formation of $H_2O_2$. The inhibition of signal B is due to the decomposition of the initially formed $H_2O_2$ by PSII particles.

Slow CL induced by PSII particles and Mn complexes, which appears in the absence of peroxidase, is not inhibited by catalase, Cyt *c*, $NaN_3$, or ethanol and thus, is initiated by triplet $O_2$. A similar CL appears in the system of luminol−$K_3Fe(CN)_6$, where the oxidation of luminol by oxygen is catalyzed by a Fe coordination complex (Rusin, 1983). Experiments (Syichev & Isak, 1990) have shown the ability of the Mn coordination complexes, including the Mn−phenanthroline complex, to catalyze the oxidation of different organic compounds by molecular oxygen. The intermediate products of the oxidation phthalhydrazides may be $H_2O_2$. It was noted that CL formation may be caused by $H_2O_2$ formed during the reaction process (without the addition of $H_2O_2$ as an initial reagent) (Rusin, 1983). Thus, the oxidation of luminol by oxygen may be accompanied by the formation of $H_2O_2$, which is detected upon an addition of peroxidase. This reaction is catalyzed by the Mn−phosphate complex, synthetic Mn complexes, and the Mn cluster of WOC. In all cases, oxidase activity appears and is maximal in the presence of 200 mM phosphate buffer, pH 8.5. Oxidase activity is implied to be the end result of the reaction, i.e., the oxidation of luminol by molecular oxygen, catalyzed by the Mn cluster. The mechanism of this reaction may differ from that characteristic to known oxidase. Earlier the catalase (Velthuys & Kok, 1978) and peroxidase (oxidation of ethanol) (Frasch & Sanders, 1988) activities of WOC were shown. Also, L-amino acid oxidase activity of cyanobacterial PSII preparations, increasing in the absence of the Mn-stabilizing protein (33 kDa) and $CaCl_2$ (Bockholt et al, 1991), and SOD activity partially connected with the Mn cluster (Ananyev et al, 1994) were established.

It is also necessary to examine the participation of hexaaquo Mn(II) in the formation of slow CL induced by PSII particles and Mn complexes. This is possible due to the destruction of the Mn cluster of WOC or Mn complexes under the conditions of the assay. However, the presence of EDTA in the reaction medium only partly inhibits the above mentioned CL. Furthermore, the addition of EDTA into the reaction medium with Tris buffer brings about the light emission. At the same time, CL induced by free Mn(II) is not observed. Thus, in cases when PSII particles and Mn complexes act as initiators of CL, Mn, which is responsible for the formation of the light emission, is coordinated.

Differences in the intensity of CL induced by Mn complexes are not related to the nuclearity of the complexes, the type of oxo-bridges between their Mn atoms or valency of Mn, but rather depend on their ligand type. Ligands (including WOC in different S states) may be ranked according to their affecting the chemical properties of Mn, determined by the ability to catalyze the oxidation of luminol by oxygen: phen > bipy > WOC ($S_3$ or $S_2$) > $HBPz_3$ > WOC ($S_{0-2}$ or $S_0$, $S_1$) > TACN. Interestingly, all ligands of the Mn complexes are heterocycles containing N atoms which interact with Mn atoms. In this way, the heterocycle will most likely be a determining factor of the oxidase activity of the Mn complexes. In the above ranking WOC is near $HBPz_3$, containing pyrazolyl which differs in structure to the imidazole group of histidine only by 2′(3′) N atom position. Accordingly, upon an $S_2-S_3$ or $S_1-S_2$ transition characteristics of the Mn cluster ligand environment change influencing the induction of luminol CL. These changes are possibly associated with the histidine residue of the D1 protein found in the ligand environment of the Mn in PSII. However, these conclusions do not exclude the oxidation of Mn nor the oxidation of histidine during the $S_2-S_3$ ($S_1-S_2$) transition.

The conclusion that the $S_3$ state rather than the $S_2$ state is responsible for the origin of signal ΔB may be supported by the data of Babcock et al. (1989) stating that $E_a$ for the $S_2-S_3$ transition (26.8 kJ/mol) significantly surpasses the $E_a$ for the $S_1-S_2$ transition (9.6 kJ/mol). Significant structural changes during the $S_2-S_3$ transition have also been proposed on the basis of a much slower reduction of the Mn cluster in the $S_3$ state than in the $S_2$ state by hydroxylamine and hydrazine (Messinger et al., 1991) and a 3-fold larger reorganization parameter for the $S_2-S_3$ transition than for the $S_0-S_1$ or $S_1-S_2$ transitions calculated from the classical Marcus electron transfer theory (Renger & Hanssum, 1992).

Interestingly, the intensity of the slow CL induced by PSII particles depends on the pH of the reaction medium significantly more than the intensity of CL induced by Mn complexes and the intensity of CL induced by additions of $H_2O_2$ (the control). Earlier it was concluded that bound $Cl^-$ in the $S_2$ state of WOC is displaced by $OH^-$ ions when the pH of the assay medium increases from 6.7 to 7.5 (Fine & Frasch, 1992). In this way, the replacement of the weak $CL^-$ ligand field by the strong $OH^-$ ligand field, influencing the electron configuration of the Mn cluster of WOC, possibly affects its oxidase activity.

In summary, this work shows that slow luminol CL induced by PSII particles is caused by $H_2O_2$ forming as a result of the oxidation of luminol by triplet molecular oxygen catalyzed by Mn clusters of WOC, which have developed oxidase activity. This reaction proceeds in the presence of 200 mM phosphate buffer, pH 8.5, or 1 M Tris-HCl, pH 8.5, with 200 $\mu$M EDTA but does not occur in the presence of only 1 M Tris-HCl. Slow CL induced by PSII particles and CL induced by synthetic Mn complexes have been performed analogously. The differences in the intensity of CL induced by WOC in $S_3$ or $S_2$ and lower S states are assumed to be connected with changes in the ligand environment of the Mn cluster upon $S_2-S_3$ or $S_1-S_2$ transition.

## ACKNOWLEDGMENT

The author expresses gratitude to Professor V. V. Klimov, Dr. G. M. Ananyev, and C. F. Davies for valuable advice and critical remarks during the preparation of this paper as well as to Professor G. C. Dismukes for presenting the synthetic manganese complexes.

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BI9514875