Active and Resting States of the O_2-Evolving Complex of Photosystem II†

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ABSTRACT: During dark adaptation, a change in the O_2-evolving complex (OEC) of spinach photosystem II (PSII) occurs that affects both the structure of the Mn site and the chemical properties of the OEC, as determined from low-temperature electron paramagnetic resonance (EPR) spectroscopy and O_2 measurements. The S_2-state multiline EPR signal, arising from a Mn-containing species in the OEC, exhibits different properties in long-term (4 h at 0 °C) and short-term (6 min at 0 °C) dark-adapted PSII membranes or thylakoids. The optimal temperature for producing this EPR signal in long-term dark-adapted samples is 200 K compared to 170 K for short-term dark-adapted samples. However, in short-term dark-adapted samples, illumination at 170 K produces an EPR signal with a different hyperfine structure and a wider field range than does illumination at 160 K or below. In contrast, the line shape of the S_2-state EPR signal produced in long-term dark-adapted samples is independent of the illumination temperature. The EPR-detected change in the Mn site of the OEC that occurs during dark adaptation is correlated with a change in O_2 consumption activity of PSII or thylakoid membranes. PSII membranes and thylakoid membranes slowly consume O_2 following illumination, but only when a functional OEC and excess reductant are present. We assign this slow consumption of O_2 to a catalytic reduction of O_2 by the OEC in the dark. The rate of O_2 consumption decreases during dark adaptation; long-term dark-adapted PSII or thylakoid membranes do not consume O_2 despite the presence of excess reductant. The EPR-detected change in the Mn site of the OEC and the decline of the O_2 consumption activity observed in PSII or thylakoid membranes occur with the same time constant. It is proposed that a structural change in the Mn site of the OEC occurs during dark adaptation, changing the electron-transport properties of the donor side of PSII and causing a conversion from an active, O_2-consuming state to a resting state incapable of O_2 consumption.

The O_2-evolving complex (OEC)[1] of photosystem II (PSII) catalyzes the four-electron oxidation of water to O_2. In the S-state model of Kok and co-workers (Kok et al., 1970; Forbush et al., 1971), successive light-induced charge separations in the PSII reaction center advance the OEC through five oxidation states, S_i, i = 0–4, with the release of an O_2 molecule being accompanied by the rapid conversion of the S_4 state to the lowest oxidation state, S_0. Dark adaptation causes the S_2 and S_3 states to decay to the S_1 state, producing a distribution of 75% S_1 and 25% S_0 shortly after continuous illumination. In the S-state model of Kok et al., it is assumed that the S_0 and S_1 states are stable in order to explain the characteristic oscillation with a period of four of the O_2 yields from a series of short flashes of light (Joliot & Kok, 1975).

Velthuys & Visser (1975) and, more recently, Vermaas et al. (1984) have shown that the S_0/S_1 ratio depends on the length of dark adaptation; the calculated S_0 concentration is nearly zero in extensively dark-adapted samples. Apparently, the PSII OEC undergoes conversions during the dark-adaptation process that stabilize the S_1 state relative to the other S states. However, the mechanism of this conversion is not understood.

There have been numerous reports of changes in the properties of the PSII OEC related to the extent of dark adaptation. More double hits (advances of two S states during a single flash of light) are observed in flash-induced O_2 yield experiments employing long-term dark-adapted samples than in experiments using briefly dark-adapted samples [see Wydryzynski (1982) and references cited therein[1] Also, extensive dark adaptation decreases the number of protons released from the OEC after the initial flash of light (Förster et al., 1981). Further, the amplitude of the 50 °C thermoluminescence band in chloroplasts, which is related to charge recombination from the S_0 and S_1 states, is strongly dependent on the length of dark adaptation, decreasing exponentially with a $t_{1/2}$ of 10 min at 25 °C (Demeter et al., 1984). Each of these results could be accounted for if the PSII OEC undergoes a structural reorganization during dark adaptation, affecting the properties of the S_0 and S_1 states. However, the molecular details of the reorganization are not revealed by any of the techniques discussed above.

In this work, we consider the nature of the change in the PSII OEC that occurs during dark adaptation. To probe the structural aspects of the change, we use low-temperature electron paramagnetic resonance (EPR) spectroscopy of the Mn site. A variety of studies [reviewed by Amesz (1983)] strongly suggest that Mn ions play a crucial role in the mechanism of photosynthetic O_2 evolution. Dismukes & Siderer (1981) showed that a multiline EPR signal from a metal ion cluster containing Mn can be produced in spinach thylakoids frozen quickly following a single laser flash and

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[1] Abbreviations: chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, O_2-evolving complex; PSI, photosystem I; PSII, photosystem II; P680, primary electron donor in PSII; Q_A, primary electron acceptor in PSII; Q_B, secondary electron acceptor in PSII; TPB, sodium tetraphenylborate; Tris, tris(hydroxymethyl)aminomethane.

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proposed that this EPR signal arises from the S_2 state. This proposal was confirmed by Brudvig et al. (1983a), who also showed that the S_2-state EPR signal can be produced by continuous illumination at 200 K of dark-adapted spinach thylakoids. We show in this paper that the intensity and hyperfine structure of the S_2-state EPR signals produced by low-temperature illumination of PSII membranes or thylakoids are strongly dependent on the length of dark adaptation, indicating that the environment of the Mn center of the OEC changes during dark adaptation.

We have also examined the effect of dark adaptation on the O_2 consumption behavior of PSII membranes and thylakoids. Sayre & Homann (1979) have reported that chloroplasts consume O_2 immediately after illumination for a period of a few minutes. We report that following this rapid phase of O_2 consumtion a slower, exponentially decaying phase of O_2 consumption occurs during dark adaptation. The decay of the slow phase of O_2 consumption and the change in the EPR properties of the Mn site occur synchronously, suggesting that these two phenomena are related. On the basis of these results, it is suggested that during dark adaptation the PSII OEC undergoes a change from an active, O_2-consuming state to a resting state incapable of O_2 consumption and that this process involves a structural change in the Mn site of the OEC.

Experimental Procedures

Thylakoids were isolated from 2-h dark-adapted market spinach leaves by the high-salt/EDTA procedure of Yocum et al. (1981) and were suspended for storage at 77 K at approximately 5 mg of chl/mL in the storage buffer, containing 20 mM MES–NaOH, pH 6.0, 15 mM NaCl, and 30% (v/v) ethylene glycol. PSII membranes were prepared by the procedure of Berthold et al. (1981), as modified by Sandusky et al. (1983), except that 5 mM EDTA and 1 g/L bovine serum albumin (Sigma fraction V powder) were added to all buffers used. All steps in the procedure were done in darkness. PSII membranes were suspended for storage at 77 K at approximately 5 mg of chl/mL in the storage buffer. Tris-washed PSII membranes were made by incubating PSII membranes (0.5 mg of chl/mL) in 0.85 M Tris-HCl (Sigma), pH 8.0, on ice and in room light for 30 min. The Tris-washed PSII membranes were resuspended after centrifugation in the storage buffer.

Chlorophyll concentrations were determined spectrophotometrically by the method of Arnon (1949). The EPR signal II spin-quantitation procedure of Babcock et al. (1983) using potassium nitrosodisulfonate as the spin standard was employed to determine the ratio of chlorophyll to PSII reaction center in PSII membrane suspensions. In the preparations used in this study, the ratio varied between 175 and 200 chl/PSII.

The low-temperature illumination method of Brudvig et al. (1983a,b) was used to generate the S_2 state for EPR study. Before dark adaptation, the samples were illuminated in a 0 or 25 °C water bath for 5 min by unfiltered, low-intensity incandescent light (100 W/m^2). After the light source was turned off, the samples were held in the water bath for a specified period of time in complete darkness. At the end of the dark-adaptation period, samples were frozen in liquid nitrogen. EPR scans of the dark-adapted samples were taken and stored on diskette by a DEC MINC-23 computer. Then, samples were removed from the EPR cavity and were placed in a nitrogen gas flow temperature-control apparatus. The sample temperature was monitored with a gold–chromel thermocouple (Air Products) calibrated with a carbon–glass resistor (Lake Shore Cryotronics) held in a sample tube. The samples were allowed to equilibrate at the chosen temperature (140–230 K) for 3 min before illumination (120 s) with white light from a 100-W quartz–halogen lamp filtered with 10 cm of water (700 W/m^2). The samples were then quickly cooled with liquid nitrogen. EPR scans of the illuminated samples were recorded in the same manner employed for the dark-adapted samples.

EPR scans were performed at 7 K on a JEOL ME-3X X-band EPR spectrometer equipped with an Oxford ESR-900 liquid helium cryostat. Sample temperatures were determined with a carbon–glass resistor (Lake Shore Cryotronics) contained in a sample tube in the sample position.

O_2 evolution and consumption measurements were made on a YSI Model 53 O_2 monitor equipped with a Teflon-membrane-covered Clark-type O_2 electrode. A Neslab RTE-9DD circulator bath was used to maintain the sample cell's water jacket at 25.0 °C and to equilibrate the temperature of the electrode and buffers before an assay. Output from the YSI O_2 monitor was digitized by the MINC-23 computer.

The O_2 consumption assay media consisted of 4.0 mL of assay buffer [20 mM MES, 15 mM NaCl, 5 mM EDTA, and 30% (v/v) ethylene glycol, pH 6.0] and 250 μM DCBQ (Eastman Kodak, recrystallized twice from 95% ethanol), to which PSII membranes ranging in concentration from 1 to 150 μg of chl/mL had been added. EDTA was present in the assay buffer to avoid complication from free metal ion catalyzed O_2 consumption [see Miles (1976)]. Thylakoids were assayed in the same medium except that 2 mM ammonium chloride was added as an uncoupler. In some assays, as noted, 100 μM TPB (Aldrich gold label) was included. Illumination for O_2 evolution/consumption assays was provided by a 1000-W quartz–halogen lamp filtered by Schott KG-5 heat-absorbing and GG-495 filters contained in a water bath (1200 W/m^2).

Two protocols were followed for illuminating samples prior to measurement of the O_2 consumption. In some assays, the sample was added to the assay medium, the O_2 electrode was positioned, and the medium was allowed to equilibrate for 5 min. Then the system was illuminated for a period of 0.5–2 min. The concentration of O_2 was measured prior to, during, and following the illumination. In other assays, the sample was added to the assay medium, allowed to equilibrate for 5 min, and then illuminated for 0.5–2 min while the electrode was kept in a temperature-regulated sample of the assay medium. Following illumination, the sample was allowed to equilibrate for a total of 4 min in the dark before positioning of the O_2 electrode and measurement of the O_2 concentration; 30 μM DCMU, 1 mM sodium ascorbate, or 3.5 mM potassium ferricyanide was added to some assays 2 min after illumination. Following illumination of the sample, all steps were done in darkness. This second protocol was followed to allow the addition of reagents after illumination and dark reequilibration and also to determine if complications arose from illumination of the O_2 electrode. When no additions were made, both protocols produced equivalent results.

Results

EPR Studies of the S_2 State. Figure 1 shows the effect of dark adaptation at 0 °C on the intensity of the S_2-state EPR signal produced by illumination of PSII membranes and thylakoids at 200 K. The EPR signal intensity produced by illumination at 200 K after 6-min incubation in the dark at 0 °C was weak (Figure 1a). Samples illuminated at 200 K after longer periods of dark adaptation (Figure 1b,c) produced much more intense S_2-state EPR signals. The 4-h dark-adapted sample produced an S_2-state EPR signal very similar

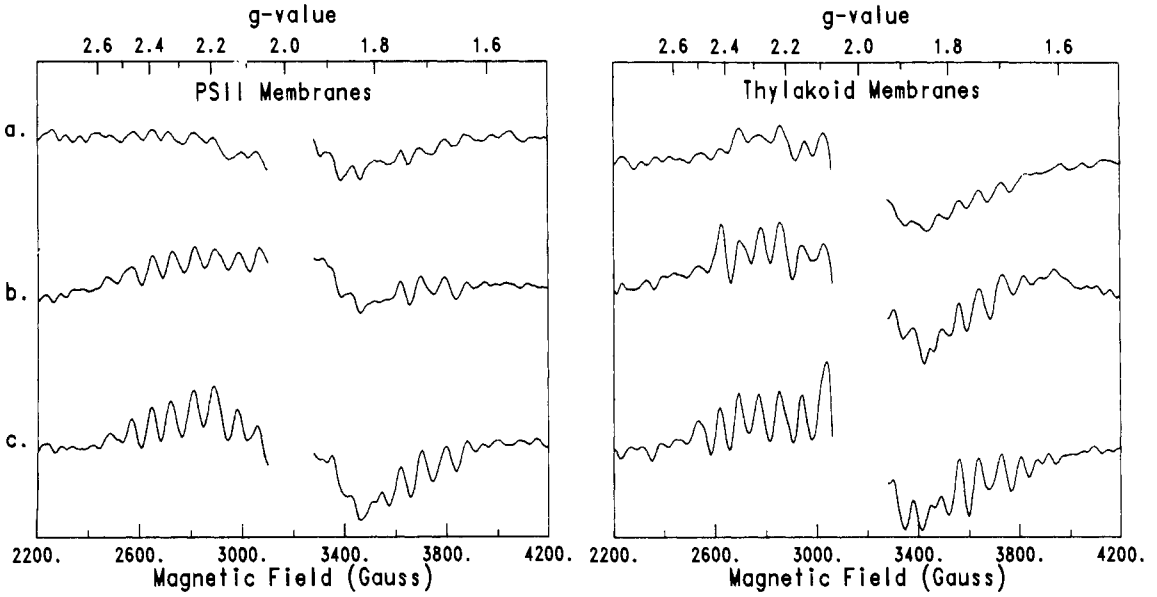

FIGURE 1: Dark-adaptation dependence of the intensity of the S_2-state multiline EPR signal produced by 200 K illumination of dark-adapted PSII membranes (left) and thylakoids (right). The spectra are the difference between the spectrum after 200 K illumination (120 s) and the spectrum of the same dark-adapted sample. The $g = 2.0$ region of the spectrum is not shown. EPR spectrometer conditions: microwave frequency 8.81 GHz, microwave power 4 mW, modulation frequency 100 kHz, and modulation amplitude 20 G. PSII membranes and thylakoids (5 mg of chl/mL) were dark adapted at 0 °C for (a) 6 min, (b) 2 h, and (c) 4 h.

to that observed by Dismukes & Siderer (1981), Brudvig et al. (1983a,b), and Hansson & Andréasson (1982). Further dark adaptation caused little change in either the intensity or the hyperfine structure of the S_2-state EPR signal. The S_2-state spectra from thylakoids showed the same dependence on dark adaptation as did PSII membranes (Figure 1). We can thus dismiss the possibility that the process occurring during dark adaptation was an abnormality induced by the Triton X-100 used in the PSII isolation procedure.

We can also exclude the possibility that the dependence of the S_2-state EPR signals on the length of dark adaptation was due to decomposition of the samples. Control trials, in which samples were incubated at 0 °C in the dark for 4 h, illuminated at 0 °C to cycle the S states, and then dark adapted for 6 min at 0 °C, subsequently produced the S_2-state EPR signal typical of short-term dark-adapted samples.

The question arises as to whether 6 min of dark adaptation at 0 °C was sufficient to allow most of the S_2 and S_3 states present during the preillumination to decay to the S_1 state. We find that the EPR spectra of samples dark adapted for 6 min at 0 °C show <10% of the multiline EPR signal associated with the S_2 state. This result indicates that the S_2 state has largely deactivated after 6 min of dark adaptation at 0 °C, in agreement with past studies on the rate of decay of the S_2 state at 0 °C (Brudvig et al., 1983a,b). Although the S_3 state decays more slowly than the S_2 state [the half-times at room temperature for deactivation of the S_2 and S_3 states in PSII membranes are approximately 20 and 100 s, respectively (Seibert & Lavorel, 1983)], incomplete S-state deactivation cannot explain the observed 3–4-fold change in the intensity of the S_2-state EPR signal between 6 min and 4 h of dark adaptation. Evidently, the ability to generate a multiline EPR signal by illumination at 200 K is affected by a change that is not related to S-state deactivation.

The dependence of the intensity of the S_2-state EPR signal on the illumination temperature was also determined. Samples were dark adapted at 0 °C either for 6 min or 4 h and then were frozen in the dark. Figure 2 shows the intensity of the multiline EPR signal induced as a function of the illumination temperature, ranging from 140 to 230 K. The 6-min dark-

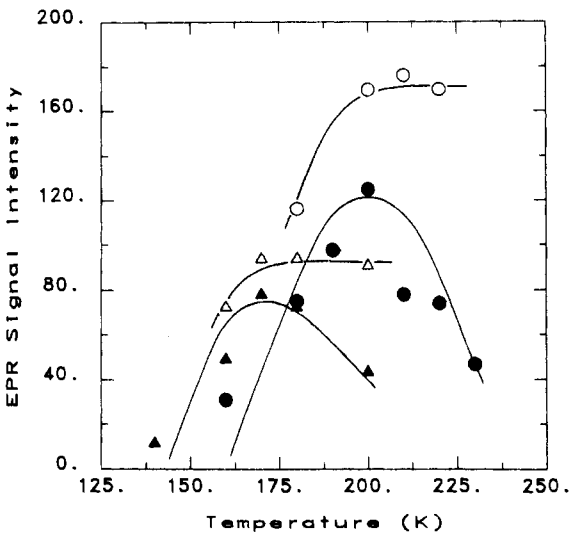

FIGURE 2: Dependence of the intensity of the S_2-state EPR signal on the illumination temperature in PSII membranes dark adapted at 0 °C for 6 min (triangles) and for 4 h (circles). Solid polygons denote untreated samples, and open polygons denote samples with 30 μM DCMU. Signal amplitudes were determined as the peak-to-peak heights of two lines to lower field and two lines to higher field of $g = 2.0$ in the light-dark difference spectrum. EPR spectrometer conditions and sample concentrations were as in Figure 1.

adapted samples produced the most intense EPR signals when illuminated at 170 K, 30 K colder than the 200 K illumination temperature required for the largest signals from the 4-h dark-adapted samples.

Previous work (Brudvig et al., 1983a; Casey & Sauer, 1984) has shown that the drop in the intensity of the S_2-state EPR signal with decreasing illumination temperature is due to a failure to photooxidize the multiline EPR signal species, although a stable charge separation occurs. The drop at higher temperature results from multiple advancement of the S states, thus producing a lower yield of the S_2 state. DCMU prevents reoxidation of the primary acceptor in PSII, Q_A, by the secondary acceptor, Q_B (Joliot & Kok, 1975). Thus, in the presence of DCMU only a single S-state advancement is al-

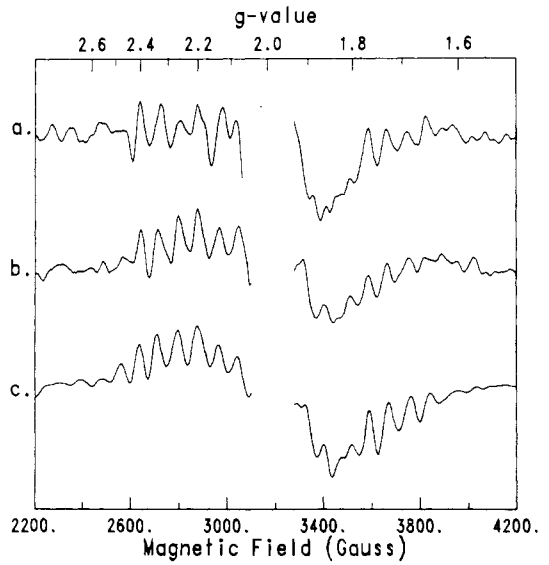

FIGURE 3: Dependence of the hyperfine structure of the S_2-state multiline EPR signal on the illumination temperature: (a) 170 K illuminated minus 6-min dark-adapted (×1.25); (b) 160 K illuminated minus 6-min dark-adapted (×1.25); (c) 200 K illuminated minus 4-h dark-adapted. Spectra are shown with different relative gains in order to allow comparison of the hyperfine structure. EPR spectrometer and sample conditions were as in Figure 1.

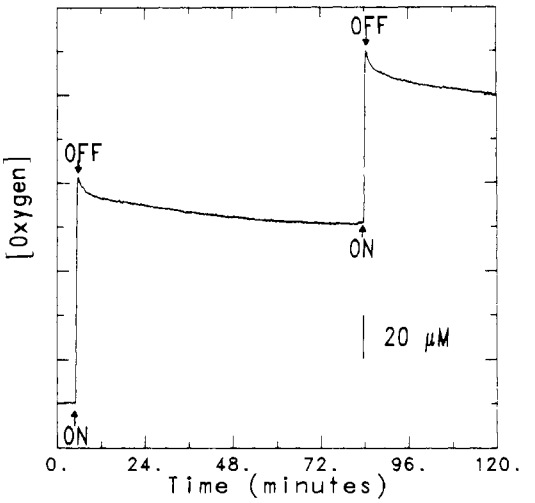

FIGURE 4: O_2 evolution and consumption trace following illumination of PSII membranes. The 4-h dark-adapted PSII membranes were assayed in the O_2 consumption assay medium at 25.0 °C at a concentration of 150 μg of chl/mL. The light was turned on and off at the marked arrows; each illumination period lasted 30 s. Immediately before the second illumination, additional DCBQ was injected into the assay chamber to give a total concentration of 500 μM (the initial DCBQ concentration was 250 μM). Except during the two illuminations, the PSII membrane suspension was maintained in complete darkness.

lowed. The following results should be obtained if the EPR signals that we observe are from the S_2 state: (i) DCMU should have no effect on the formation of the multiline EPR signal by continuous illumination in the low-temperature range, and (ii) DCMU should inhibit the drop in signal intensity at higher temperatures. When 30 μM DCMU as added after preillumination and either 6-min or 4-h dark adaptation, *both* of these expectations were realized (Figure 2). DCMU also had no effect on the dark-adaptation process. Identical results were obtained from 4-h dark-adapted samples regardless of whether 30 μM DCMU was added 2 min after illumination or 4 h after illumination.

The results obtained when DCMU was present show that photooxidation of the Mn site occurs more readily at lower temperature in 6-min dark-adapted samples than in 4-h dark-adapted samples. In addition, the 6-min dark-adapted samples have a reduced temperature threshold for multiple S-state advancements. Multiple S-state advancements require that multiple electron transfers occur on both the donor and acceptor sides of PSII. Thus, the lower temperature for multiple S-state advancements in 6-min dark-adapted samples could be explained either by a lower temperature onset for oxidation of the S_2 to the S_3 state or by a lower temperature onset for electron transfer between Q_A and Q_B, depending on which step is rate limiting under these conditions.

The large difference in the optimal temperatures for production of the S_2-state EPR signal in 6-min and 4-h dark-adapted samples explains the increase in the signal intensity observed during dark adaptation when 200 K illumination was used, as in Figure 1. As the dark adaptation proceeds, the average optimal temperature for photooxidation of the S_2 state in a sample moves from 170 to 200 K.

In addition to a different dependence on illumination temperature between 6-min and 4-h dark-adapted samples, we also observe a different line shape of the S_2-state EPR signal. The 4-h dark-adapted samples produced an S_2-state EPR signal (Figure 3c) quite similar to those previously reported (Dismukes & Siderer, 1981; Brudvig et al., 1983a,b), and the line shape of this EPR signal was independent of the illumination temperature. In contrast, the line shape of the S_2-state EPR signal produced in 6-min dark-adapted samples depended on the illumination temperature. Illumination at 160 K produced an EPR signal (Figure 3b) similar to that in the 4-h dark-adapted sample. However, illumination of the 6-min dark-adapted sample at 170 K produced an EPR signal (Figure 3a) with a different hyperfine pattern and with resonances spanning a wider field range.

O_2 Consumption by PSII Membranes. The EPR results in the previous section suggest that a change in the environment of Mn in the OEC occurs during dark adaptation. Is this change reflected in the catalytic properties of the PSII OEC? To investigate this question we have examined the O_2 evolution/consumption properties of PSII membranes and thylakoids.

Figure 4 shows the O_2 evolution/consumption behavior of untreated PSII membranes at 25.0 °C. O_2 was evolved when the PSII membrane suspension was illuminated. The light-saturated rate was between 450 and 800 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}, depending on the preparation, although a nonsaturating light intensity was used in Figure 4. Immediately after the lamp was turned off, a biphasic consumption of O_2 was observed. The fast phase of O_2 consumption lasted about 2 min and had a variable maximum rate of up to 30 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}. A slow phase of O_2 consumption followed the fast phase, lasting for approximately 1 h following illumination.

The fast phase of O_2 consumption following illumination of untreated PSII membranes was strongly dependent on the length and intensity of the illumination period, suggesting that dissipation of reactive intermediates formed during illumination caused the initial rapid consumption of O_2. The magnitude of this fast phase of O_2 consumption was quite variable and nearly absent in some preparations. Moreover, the fast phase of O_2 consumption showed an inverse correlation with O_2 evolution activity. We shall not discuss this reaction further.

The maximum rate of O_2 consumption (μmol/h) in the slow phase was directly proportional to the amount of the PSII OEC in the assay suspension and was equal to 0.43 ± 0.10 μmol

of O_2 (mg of chl)$^{-1}$ h^{-1} (based on the slope of a plot of rate vs. amount of chl in the assay, data not shown). This maximum rate was observed only after a saturating amount of light was given to the sample. In these experiments, 30 s of illumination (1200 W/m^2) resulted in a maximal rate of the slow phase of O_2 consumption reaction if the concentration was $\leq$50 μg of chl/mL. The maximum rate of O_2 consumption in the slow phase was also proportional to the maximum rate of O_2 evolution under light-saturating conditions. These results suggest that the slow phase of O_2 consumption is a property of the PSII OEC.

Possible sources of O_2 consumption not associated with PSII were also investigated. We can rule out diffusion of O_2 from the assay chamber as the source of decrease in O_2 concentration. If diffusion of O_2 caused the decrease in O_2 concentration following illumination, then we would expect the concentration of O_2 to approach the equilibrium value asymptotically. However, Figure 4 shows that the O_2 concentration decreased to a limiting concentration significantly above the equilibrium O_2 concentration. Furthermore, injection of air-saturated ethanol into the assay chamber to induce an increase in the O_2 concentration showed that diffusion of O_2 out of the assay chamber together with the slow consumption of O_2 by the electrode proceeded at a constant rate much less than our measured rate of O_2 consumption by PSII membranes throughout the time period of our measurements. We conclude that the slow phase of O_2 consumption derives from a light-initiated process occurring in the PSII membrane sample.

There are several possible components present in the PSII membrane assay suspension that could catalyze O_2 consumption. Free metal ions such as Mn^{2+} have been found to catalyze O_2 reduction in PSII (Miles, 1976). However, this reaction is suppressed in the presence of EDTA (Miles, 1976). The slow phase of O_2 consumption that we observe after illumination of PSII membranes was not affected by EDTA, and therefore, we can exclude free metal ions as the source of the slow phase of O_2 consumption. PSI also is known to reduce O_2 photochemically (Mehler, 1951). However, interference by O_2-consuming reactions involving PSI can be discounted because our preparations had negligible PSI content as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and because illumination of our preparation at 10 K did not induce any EPR signal I ($P700^+$).

It is also possible that an unstable intermediate was produced photochemically and slowly reacted with O_2 in the dark until it was completely consumed. Sayre & Homann (1979) have reported that illumination of PSII in the presence of agents that accelerate the deactivation of the S states (ADRY reagents) leads to O_2 consumption with the production of hydrogen peroxide. This reaction was observed to persist for a few minutes after illumination. However, we found that the addition of 0.1 mg/mL catalase or superoxide dismutase did not alter the slow phase of O_2 consumption. The fast phase of O_2 consumption that we noted above probably corresponds to the light-induced O_2 consumption reported by Sayre & Homann (1979). The results of experiments done with added ascorbate or ferricyanide (see below) further argue against the photochemical production of an unstable intermediate as a source of the slow phase of O_2 consumption. Addition of ascorbate *after* illumination *increased* the initial rate of O_2 consumption, which would not be expected if a photochemically produced reactive intermediate led to the consumption of O_2.

To determine if the OEC causes the slow phase of O_2 consumption, we examined the O_2 consumption behavior of

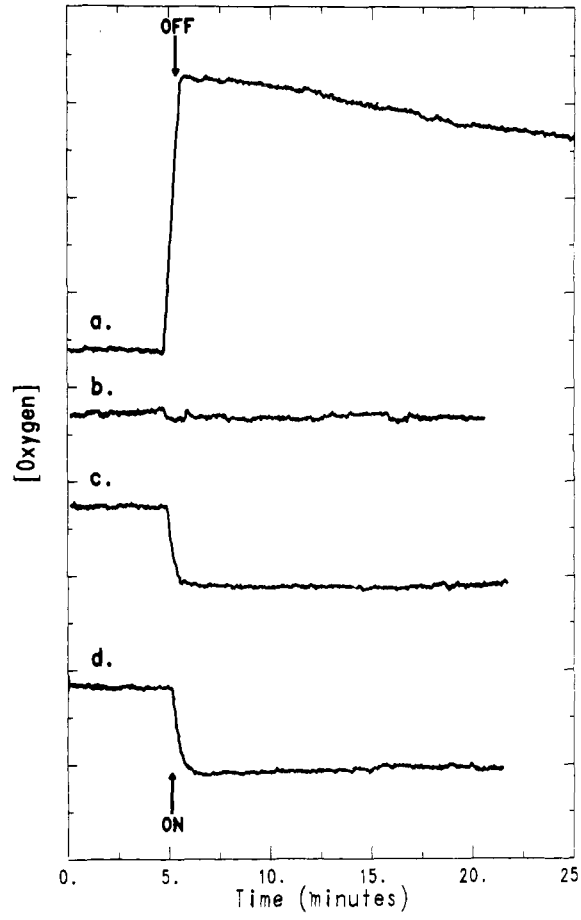

FIGURE 5: O_2 evolution and consumption traces following illumination of PSII membranes. The conditions were as in Figure 4 except as noted: (a) untreated PSII membranes; (b) Tris-washed PSII membranes containing 100 μM *sym*-diphenylcarbazide as an artificial electron donor; (c) untreated PSII membranes plus 100 μM TPB; (d) Tris-washed PSII membranes plus 100 μM TPB. In all four traces, the PSII membranes were assayed at 20 μg of chl/mL. The traces have been corrected for the background O_2 consumption rate under each set of conditions. The traces are arbitrarily spaced vertically with a constant O_2 concentration scale.

Tris-washed PSII membranes. Tris washing causes O_2 evolution activity to be lost through release of Mn and three polypeptides but does not inhibit light-driven electron transport in PSII provided that an exogenous electron donor is supplied [see Amesz (1983) and references cited therein]. Curves a and b of Figure 5 compare the response of untreated PSII membranes with Tris-washed PSII membranes. Tris-washed PSII membranes did not consume O_2 following illumination in the presence of 250 μM DCBQ and 100 μM *sym*-diphenylcarbazide, an electron donor that functions if water oxidation is impaired (Kimimura & Katoh, 1972). However, the Tris-washed PSII membranes were capable of high rates of electron transport; the Tris-washed sample had an electron transport rate of 680 μequiv (mg of chl)$^{-1}$ h^{-1} as measured by the photoreduction of 2,6-dichloroindophenol with *sym*-diphenylcarbazide as the electron donor.

The behavior of Tris-washed PSII membranes in the presence of TPB further substantiates the involvement of the OEC in the slow phase of O_2 consumption. TPB acts as a preferred electron donor to P680 (Erixon & Renger, 1974; Homann, 1972) but allows the acceptor side of PSII to function normally. When TPB is added to untreated samples, O_2 evolution is inhibited but can be restored by removal or complete photooxidation of the TPB (Erixon & Renger, 1974). Erixon & Renger (1974) also showed that TPB acts as an

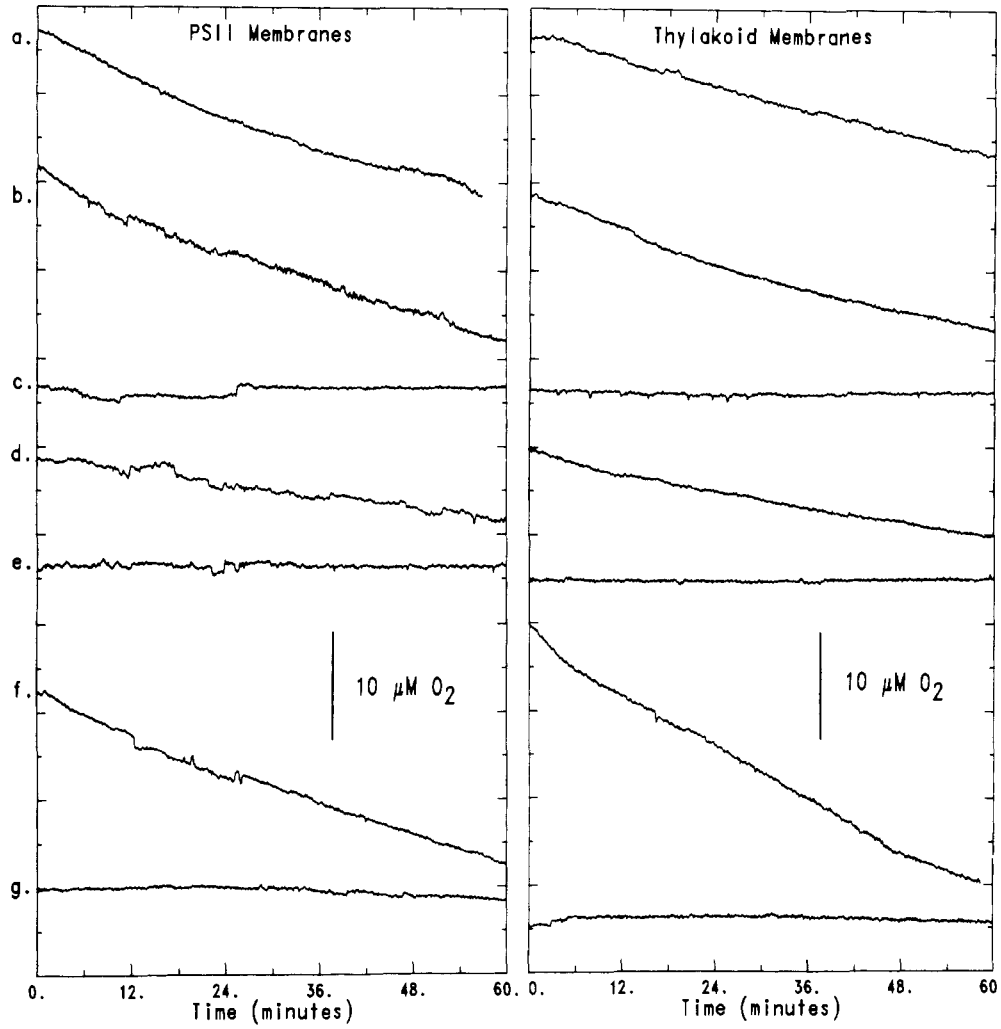

FIGURE 6: O_2 consumption by PSII membranes (left) and thylakoids (right). Samples were dark adapted for 4 h at 0 °C before the assays, and except for the illumination, all steps were done under a green safe light. The chlorophyll concentration was 150 μg/mL in all cases. The 30-s illuminations were performed without the electrode in position, and the zero of time was 4 min after the end of the illumination. The samples were assayed at 25.0 °C in the O_2 consumption assay medium under the following conditions: (a) 30 μM DCMU was added 2 min after illumination; (b) no additions after illumination; (c) no additions and without preillumination; (d) 3.5 mM ferricyanide was added 2 min after illumination; (e) 3.5 mM ferricyanide was added without preillumination; (f) 1 mM ascorbate was added 2 min after illumination; (g) 1 mM ascorbate was added without preillumination. The traces have been corrected for the background O_2 consumption rate under each set of conditions. The traces are arbitrarily spaced vertically with a constant O_2 concentration scale.

electron donor in Tris-washed chloroplasts. Curves c and d of Figure 5 show that both untreated and Tris-washed PSII membranes consumed O_2 when illuminated in the presence of TPB at rates of 65 and 42 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}, respectively, in agreement with the results of Sayre & Homann (1979). Following illumination in the presence of TPB, both untreated and Tris-washed PSII membranes consumed O_2 in the dark for approximately 2 min; after that, the base-line rate of O_2 consumption resumed. Only when untreated PSII membranes were assayed in the absence of TPB did we observe rates of O_2 consumption greater than the base-line rate at times longer than 2 min in the dark. This result indicates that the slow phase of O_2 consumption occurred only when a functional OEC was present *and* after the OEC was cycled through the S states during illumination. Therefore, we assign the slow phase of O_2 consumption to a reaction involving the OEC.

Figure 4 shows that the rate of O_2 consumption in the slow phase decreased during dark adaptation; after about 1 h the rate had decreased to the rate observed before illumination. When the O_2 consumption rate had returned to the preillumination value, subsequent illumination caused a repeat of the O_2 evolution/consumption pattern (Figure 4) provided that additional DCBQ was added before the second illumination. The additional DCBQ was required to observe the maximal rate of O_2 evolution in the second illumination because the initial DCBQ was substantially reduced during the first illumination and was not significantly reoxidized during the dark period. If 3.5 mM ferricyanide was included in the assay, then additional DCBQ was not required to observe the maximal O_2 evolution activity in the second illumination. The fact that nearly the same O_2 evolution/consumption pattern could be repeated in a given PSII membrane assay suspension shows that the decrease in the rate of O_2 consumption during dark adaptation is not due to denaturation of the PSII membranes during the 25 °C dark-incubation period. The decrease in the rate of O_2 consumption catalyzed by the OEC must either be due to depletion of the supply of electrons used in the reduction of O_2 or to a mechanism changing the OEC to a form incapable of O_2 consumption.

To investigate the source of electrons used in the reduction of O_2, we have examined the effect of ascorbate and ferricyanide on the slow phase of O_2 consumption. The addition of 1 mM ascorbate increased the initial rate of O_2 consumption slightly (compare curves f and b of Figure 6). Note that prior illumination of the assay mixture was required in order to observe the slow phase of O_2 consumption in the presence of

ascorbate (compare curves g and f of Figure 6). It is clear from this result that the decrease in rate of the slow phase of O_2 consumption during dark incubation cannot be explained by depletion of reductant; even with excess reductant, long-term dark-adapted samples did not consume O_2. Adding 3.5 mM ferricyanide significantly decreased the initial rate of O_2 consumption (Figure 6d). In all cases, thylakoids exhibited O_2 consumption properties similar to those of PSII membranes (Figure 6, right-hand side). The results of the experiments done in the presence of either ascorbate or ferricyanide suggest that the reduced endogenous plastoquinone pool or added DCBQ served as the source of electrons for the slow phase of O_2 consumption in PSII membranes following illumination.

The amount of O_2 consumed by PSII membranes in the slow phase of O_2 consumption depended on the amount of reductant available. The total number of molecules of O_2 reduced per PSII reaction center during the period immediately after illumination until the base-line rate of O_2 consumption was restored was 72 with excess reductant (1.0 mM ascorbate added after illumination), 49 with only the DCBQ photoreduced after 30 s of illumination available as a reductant, and <10 with 3.5 mM ferricyanide added after illumination. Except when ferricyanide was added, whenever the membrane suspension was illuminated long enough to induce O_2 evolution, the slow phase of O_2 consumption followed. However, as noted above, a saturating amount of light was required to observe the maximum rate of the slow phase of O_2 consumption.

Our results suggest that reduced endogenous plastoquinone or reduced DCBQ served as the electron donor for the slow phase of O_2 consumption and that the OEC catalyzes the reduction of O_2. What is the pathway of electron transfer from the electron donor to the catalytic site? It is possible that electrons could retrace the pathway of photoassisted electron transfer from water to Q_B, as is suggested by thermoluminescence experiments (Rutherford et al., 1982). However, we found that DCMU did not inhibit the slow phase of O_2 consumption (compare curves a and b of Figure 6). DCMU is known to inhibit electron transfer in the forward direction, but it is possible that DCMU does not inhibit electron transfer in the reverse direction. In this regard, *o*-phenanthroline, which inhibits electron transfer between Q_A and Q_B in bacterial reaction centers, does not inhibit electron transfer in the reverse direction when added after flash illumination because it binds poorly when Q_B is singly reduced (Wraight & Stein, 1980). Thus, the possibility remains open that the pathway of electron transfer from the reductant to the catalytic site involves the Q_B site.

Correlation of O_2 Consumption and EPR Studies. Both the EPR spectra of Mn in the OEC and the slow phase of O_2 consumption exhibited changes during dark incubation. Are these two phenomena related? To compare the two sets of data, we first set up a model to interpret the data. In this model, we propose that the PSII OEC undergoes a conversion in the dark from an active state, A, to a resting state, R, with a first-order rate constant, k_R. We also propose that only the active state is able to consume O_2 in the dark and that a reduced quinone, QH_2, serves as the ultimate electron and proton donor (eq 1). Both the EPR and O_2 consumption data

$$O_2 + 2QH_2 + A \xrightarrow{k} 2H_2O + 2Q + A \qquad (1)$$

can be related to the fraction of the sample in the active state, and this fraction should decay exponentially from a value of 1.0 immediately after illumination to 0.0 after long dark incubation. A plot of ln (fraction active) vs. time should be linear with a slope equal to $-k_R$ according to the above model.

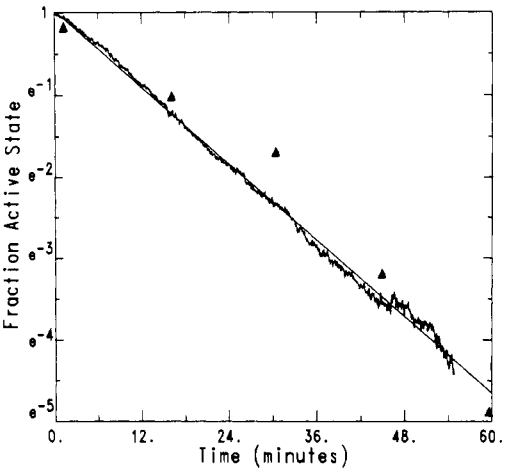

FIGURE 7: Comparison of the multiline EPR signal intensity obtained by 200 K illumination of PSII membranes as a function of dark-adaptation time (triangles) with the decrease in O_2 concentration after illumination of PSII membranes (solid line) at 25.0 °C. The O_2 consumption trace was obtained under conditions as in Figure 6b. See text for the formulas used to calculate the fraction active from EPR and O_2 consumption data. The straight line drawn through the data is a linear least-squares fit to the O_2 consumption data. Both sets of data are consistent with a first-order, active to resting state decay with half-times of 30 ± 2 (O_2 consumption data) and 31 ± 5 min (EPR data) at 25 °C.

The intensity of the S_2-state EPR signal, $I(t)$, observed with 200 K illumination, as in Figure 1, is taken to be proportional to [R], since only a small S_2-state EPR signal was induced by 200 K illumination of short-term dark-adapted samples. Hence, the fraction of the sample in the active state will be

$$\text{fraction active} = [A(t)]/[A(0)] = 1 - I(t)/I_{max} \qquad (2)$$

where I_{max} is the maximum S_2-state EPR signal intensity observed after long dark incubation.

The rate of change of O_2 concentration with time, $[O_2(t)]'$, is given by

$$[O_2(t)]' = -k[O_2(t)][QH_2(t)]^2[A(t)] \qquad (3)$$

When excess reductant is present, as in the case when 1 mM ascorbate is added or when sufficient preillumination is provided, only a small fraction of the reductant is oxidized during the dark incubation period, and hence, $[QH_2(t)] \sim [QH_2(0)]$. In addition, the change in O_2 concentration is much less than the total O_2 concentration. In this case, integration of eq 3 and rearrangement gives

$$\text{fraction active} = 1 - ([O_2(0)] - [O_2(t)])/([O_2(0)] - [O_2(\text{final})]) \qquad (4)$$

where $[O_2(\text{final})] = [O_2(0)](1 - k[A(0)][QH_2]^2/k_R)$.

Figure 7 shows the comparison of the fraction of the sample in the active state as determined with either eq 2 (EPR data) or 4 (O_2 consumption data) as a function of time at 25 °C. The agreement of the two sets of data provides support for the model presented and, moreover, shows that the change in the EPR properties and O_2 consumption occur synchronously.

DISCUSSION

We have presented data in this paper that indicate dark adaptation causes a conversion of the PSII OEC from an active state able to consume O_2 to a resting state incapable of O_2 consumption. This change during dark adaptation affects electron transport in the PSII OEC, changing the temperature dependence of the S_1- to S_2-state transition. Moreover, the change during dark adaptation affects the environment of the Mn site in the OEC such that the line shape of the S_2-state EPR signal is different in the active and resting states. In

6-min dark-adapted samples, the line shape of the S_2-state EPR signal depends on the illumination temperature used to generate the S_2 state, whereas, in 4-h dark-adapted samples, the line shape of the S_2-state EPR signal is independent of the illumination temperature.

There are two points of particular interest with regard to the properties of the Mn site in the OEC. The first is that the Mn site apparently can be photooxidized at lower temperature in 6-min dark-adapted samples than in 4-h dark-adapted samples. The apparent lower activation barrier for the S_1- to S_2-state transition in active-state samples could be accounted for if the Mn site undergoes a structural change during the dark-adaptation process. Such a structural change could also account for the different S_2-state EPR signals observed in active vs. resting-state samples. A structural change could also account for the change in O_2 consumption activity between the active and resting states (see below).

The second point of interest is that the S_2-state EPR signal produced in active-state samples depends on the illumination temperature. The fact that we observe a different line shape for the S_2-state EPR signal in active-state samples illuminated above 170 K suggests that a rearrangement occurs in the Mn site in active-state samples between 160 and 170 K. Such a rearrangement is apparently blocked in resting-state samples. Another interpretation of the results is that there are two distinct Mn sites, each producing a distinct multiline EPR signal and that these two sites are connected by an electron-transport path that is blocked at temperatures below 170 K in the active state. A third interpretation is that a second site is photoxidized in the active state by illumination above 170 K and that oxidation of this site perturbs the site giving rise to the multiline EPR signal. This third possibility appears to be less likely because experiments done in the presence of DCMU, which prevents more than one charge separation, yield the same result as experiments done on untreated samples. All three possibilities imply that the properties of the PSII OEC are different in the active and resting states.

The active state of the OEC is able to catalyze the reduction of O_2. One could imagine several schemes by which the OEC reduces O_2. One is reverse turnover of the OEC in which O_2 is reduced to water. Such a reaction is thermodynamically favorable, and many enzymes are known to catalyze both forward and reverse directions of a reaction. A reverse turnover cycle could be initiated if the S_0 and S_4 states are in equilibrium. For example, the binding of O_2 to the S_0 state could lead to the formation of the S_4 state. An equilibrium between S_4 and S_0 must favor the S_0 state, in view of the results of studies of O_2 yields after flashes (Joliot & Kok, 1975), but the small fraction of S_4 in equilibrium with S_0 could back-react to the S_1 state by using known reaction paths. Such a reverse turnover of the OEC could maintain a steady-state population of S_1 via interaction of S_0 with O_2, as was previously postulated by Kok et al. (1975). It is also possible that O_2 could be partially reduced by the OEC, leading to superoxide or peroxide production. In these cases, one need not postulate reverse turnover of the OEC. However, the lack of effect of catalase and superoxide dismutase on the slow phase of O_2 consumption by PSII membranes argues against the release of substantial superoxide or peroxide from the OEC in the dark. The possibility of a reverse-turnover mechanism for O_2 reduction is most attractive because it suggests that the same forms of O_2/H_2O are stabilized by the OEC whether a forward, oxidizing cycle or a reverse, reducing cycle is being driven.

It is probable that the rate of electron and/or proton transfer to the OEC limits the rate of O_2 reduction. One estimate of the rate of electron transfer to the OEC is provided by the rate of deactivation of the S_2 and S_3 states; the half-times for deactivation of the S_2 and S_3 states in PSII membranes at 25 °C are 20 and 100 s, respectively (Seibert & Lavorel, 1983). It is unlikely that O_2 reduction could proceed at a rate significantly faster than the rate of S-state deactivation, although we note that past measurements of S-state deactivation have been made under conditions where the amount of reductant probably limited the rate of S-state deactivation. Our observed turnover numbers and initial rate of O_2 consumption are in line with the values predicted on the basis of S-state deactivation rates.

Vermaas et al. (1984) were able to observe the evolution of O_2 after three flashes of light were given to extensively dark-adapted thylakoids. This result shows that the resting state releases O_2 upon reaching the S_4 state. Thus, the light-induced stepwise forward advance of the S states is not inhibited in the resting state. The proton-release experiments of Förster et al. (1981), however, suggest that the resting state is less protonated than the active state. A possible model that explains our data, as well as the results described above, is as follows. The Mn site in the resting state could be the μ-oxo-bridged Mn dimer proposed by Dismukes & Siderer (1981). This structure was used as a basis for interpeting the S_2-state EPR spectrum. Oxidation of a μ-oxo-bridged Mn dimer after a flash of light would not be expected to release protons but would advance the net oxidation state of the Mn dimer complex. The blockage of O_2 consumption by the OEC in the resting state could be attributed to a relative inertness to ligand exchange of the μ-oxo-bridged structure. The active state, in contrast, could catalyze O_2 reduction if the H_2O or O_2 binding site were able to exchange exogenous ligands freely. Thus, the light-induced resting to active state change in the OEC may involve a reorganization of the coordination sphere of Mn. The transformation from the resting to active state could be triggered by the release of O_2 from the substrate binding site upon illumination.

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Lactose Repressor Protein Modified with Dansyl Chloride: Activity Effects and Fluorescence Properties†

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ABSTRACT: Chemical modification using 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride) has been used to explore the importance of lysine residues involved in the binding activities of the lactose repressor and to introduce a fluorescent probe into the protein. Dansyl chloride modification of *lac* repressor resulted in loss of operator DNA binding at low molar ratios of reagent/monomer. Loss of nonspecific DNA binding was observed only at higher molar ratios, while isopropyl β-D-thiogalactoside binding was not affected at any of the reagent levels studied. Lysine residues were the only modified amino acids detected. Protection of lysines-33 and -37 from modification by the presence of nonspecific DNA correlated with maintenance of operator DNA binding activity, and reaction of lysine-37 paralleled operator binding activity loss. Energy transfer between dansyl incorporated in the core region of the repressor protein and tryptophan-201 was observed, with an approximate distance of 23 Å calculated between these two moieties.

The lactose repressor protein regulates the transcription of the *lac* metabolizing enzymes in *Escherichia coli* by specific interaction at the operator site in the genome (Miller & Reznikoff, 1980). This binding is modulated by the presence of sugar ligands bound to the tetrameric repressor protein (M_r ~150 000); inducers decrease the affinity of the protein for operator DNA, while antiinducers stabilize the repressor–operator complex (Miller & Reznikoff, 1980). The interaction of operator DNA with repressor protein is salt dependent, and six to eight ion pairs have been estimated to participate in complex formation (Record et al., 1977; Barkley et al., 1981; Winter & von Hippel, 1981). Thus, positive charges on amino acid side chains in the repressor would be expected to play a role in binding of the protein to the polyacidic backbone of the target DNA. The importance of specific lysine residues has been suggested by genetic (Miller, 1979) and recent chemical studies (Whitson et al., 1984). Two domains within the repressor, the NH_2-terminus (amino acids 1–59) and the core protein (amino acids 60–360), can be isolated by mild proteolytic digestion (Platt et al., 1973; Geisler & Weber, 1977). The NH_2-terminus binds to nonspecific DNA and operator-containing DNA (Jovin et al., 1977; Ogata & Gilbert, 1979), while the core protein binds to inducer and to operator DNA (Platt et al., 1973; Matthews, 1979). The two tryptophans in the repressor monomer are located in the core domain of the protein (Beyreuther et al., 1975); an amber mutation has been isolated which can be suppressed in the appropriate bacterial strain with consequent introduction of a tyrosine at the site normally occupied by Trp-220 (Sommer et al., 1976). With this mutant protein, any energy transfer to other fluorescent moieties must be from Trp-201. In this study, we have reacted the repressor molecule with 5-(dimethyl-

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