Generation, Oxidation by the Oxidized Form of the Tyrosine of Polypeptide D2, and Possible Electronic Configuration of the Redox States S_0, S_{-1}, and S_{-2} of the Water Oxidase in Isolated Spinach Thylakoids†

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ABSTRACT: Suitable treatment of thylakoids with hydrazine permits a high population of the redox states S_0, S_{-1}, and S_{-2} in the water oxidase. Experiments performed with dark-adapted samples enriched either in the oxidized or reduced form of the redox-active tyrosine, Y_D, of polypeptide D2 reveal that Y_D^{ox} is a unique endogenous oxidant within the PS II complex which causes a one-electron abstraction from the water oxidase in states S_0, S_{-1}, and S_{-2}, respectively. A kinetic analysis of the period four oscillation of oxygen yield induced by a train of short flashes in dark-adapted samples permits the determination of the rate constants of electron abstraction from the reduced water oxidase by Y_D^{ox}. A value of 9×10^{-4} s^{-1} was found for the oxidation of S_0 and S_{-2}, while S_{-1} becomes oxidized with a rate constant of 4×10^{-4} s^{-1} at 20 °C and pH 7.2. The redox state S_0 generated either from S_1 via the three-flash-induced oxidative pathway through S_4 or from a one-flash oxidation of the S_{-1} state obtained by S_1 reduction with NH_2NH_2 exhibits the same kinetics as S_0 oxidation by Y_D^{ox}. On the basis of these findings and data taken from the literature, the electronic configuration of the manganese atoms in the tetranuclear cluster is discussed. It is assumed that the dimer model of two binuclear manganese groups within the tetranuclear cluster comprises a functional heterogeneity: (i) one binuclear center, referred to as the catalytic group, is proposed to be involved in the oxidative pathway leading to the eventual oxidation of water to dioxygen, and (ii) the other binuclear center, symbolized as component C, is redox-inert during the oxidative pathway but can be reduced by exogenous (and endogenous?) components, thereby forming the states S_{-1} and S_{-2} of the water oxidase. An asymmetric protein matrix around the tetranuclear manganese cluster is assumed to be responsible for the functional heterogeneity of the manganese centers.

Photosynthetic water oxidation to dioxygen coupled with the release of four protons into the thylakoid lumen takes place within a manganese-containing functional unit (referred to as the water oxidase) which is a part of the PS II complex integrated into the membrane. The overall process comprises a sequence of univalent electron-transfer steps which continue until after the accumulation of four oxidizing redox equivalents dioxygen is released. This highly endergonic reaction sequence, referred to as the Kok cycle (Kok et al., 1970), is energetically driven by $P680^+$ as the oxidant, formed during the primary step of light-induced charge separation within the PS II reaction center [for a review, see Renger (1992)]. A redox-active amino acid residue (Tyr of polypeptide D1; Debus et al., 1988a; Metz et al., 1989), referred to as Y_Z, acts as an intermediate electron carrier which functionally connects the water oxidase with $P680^+$. Although the kinetics of the elementary reactions in the overall reaction sequence of water oxidation have been resolved in great detail by various spectroscopic techniques [for recent reviews, see Babcock (1987), Renger (1987a), Renger and Wydrzynski (1991), and Rutherford et al. (1992)], key questions on the structure and mechanism are still far from being satisfactorily answered [for a list, see Renger (1987b)].

With respect to the structure of the water oxidase, progress has been achieved by spectroscopic techniques (EPR, EXAFS), leading to models of a cluster of four spin-coupled manganese centers (Kim et al., 1992; Kusunoki, 1992a,b; Zheng & Dismukes, 1992) with a special arrangement of μ-oxo- and μ-carboxylato-bridged manganese atoms (Kim et al., 1990; Yachandra et al., 1992; Debus, 1992). However, little information is available on the structure of the ligand sphere established by the protein matrix and the nature of the polypeptides that provide direct ligation of the manganese cluster [for a detailed discussion, see Debus (1992)]. In referene to the mechanism, the entry of substrate water into the redox cycle (and its mode of coordination) is an unresolved problem. Likewise, questions on the nature of the oxidizing equivalents (metal- or ligand-centered electron abstraction) in the different redox states S_i and the mode of oxygen–oxygen bond formation (probably at the level of a peroxidic configuration) remain to be answered. Consensus exists that the redox transition $S_1 \rightarrow S_2$ is a metal-centered oxidation [(Mn(III) → Mn(IV)], but the nature of the other redox steps remains to be clarified. Latest XANES experiments might suggest that all oxidation steps are metal-centered reactions up to the redox state S_3 (Ono et al., 1992), but this conclusion cannot be considered as straightforward (Kusunoki et al., 1992a). In respect to the substrate water chemistry, it is widely assumed that its oxidation takes place only after, or concomitantly with, the last univalent electron abstraction leading to S_4 [for a review, see Rutherford et al. (1992)]. However, it must be emphazised that unambiguous experimental proof for this assumption is still lacking (Renger, 1987b). Recently, a disturbed water oxidase was shown to generate a peroxidic state without forming S_4 and dioxygen

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(Ananyev et al., 1992). It remains to be clarified whether an analogous reaction also takes place as an intermediary step within the oxidative pathway from water to dioxygen.

In addition to its key role in the water oxidase, the manganese cluster also exhibits a catalase-like activity (Velthuys & Kok, 1978; Frash & Mei, 1987; Mano et al., 1987). A specific manipulation of the redox state population can be achieved by NH_2OH and NH_2NH_2 (Bouges, 1971; Kok & Velthuys, 1977). These compounds, which are isoelectronic with H_2O_2, not only reductively dissipate oxidizing redox equivalents within the water oxidase with a very unusual dependence of the rate on the redox state S_i (Messinger et al., 1991) but also form additional redox states referred to as S_{-1}, S_{-2}, and S_{-3} (Bouges, 1971; Kok & Velthuys, 1977; Beck & Brudvig, 1988; Renger et al., 1990; Messinger et al., 1991). The generation of "superreduced" redox states offers an additional possibility for analyzing the reactivity of the water oxidase.

This article describes the generation of redox states S_0, S_{-1}, and S_{-2} and their interaction with the oxidized form of the redox active tyrosine of polypeptide D2 (Debus et al., 1988b; Vermaas et al., 1988), symbolized by Y_D. In its reduced form, Y_D causes fast S_2 and S_3 decay with kinetics in the time domain of seconds, while the oxidized form (Y_D^{ox}) slowly (tens of minutes) transforms S_0 into S_1. The data from this study show that S_0 and S_{-2} exhibit virtually identical univalent reoxidation kinetics, while the S_{-1} transition to S_0 is markedly slower. The implications of these findings will be discussed.

MATERIALS AND METHODS

Isolated thylakoids were prepared from market spinach (Winget et al., 1965) with modifications described in Messinger (1993). After the final isolation step, the preparations were resuspended in the freezing medium (buffer A) at pH 6.5, containing 400 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 10 mM Mes/NaOH, to chlorophyll concentrations of about 4 mg/mL. For storage, the samples were frozen in small aliquots in liquid nitrogen and then kept at −80 °C.

After a very long storage time of several months at −80 °C, thylakoids are enriched in the reduced form of Y_D (Messinger & Renger, 1990; Vass et al., 1990). These preparations which still contain a high level of S_1 (see Results) will be referred to as "S_1Y_D"-thylakoids. "$S_1Y_D^{ox}$"-thylakoids were prepared from S_1Y_D-thylakoids by preillumination with one flash at 0 °C and subsequent dark incubation as described previously (Messinger et al., 1991).

Dark-adapted thylakoids with a comparatively high population of the lower redox states S_0, S_{-1}, and S_{-2} were obtained by a treatment with NH_2NH_2, whereas our attempts to accumulate a "stable" S_{-3} state were unsuccessful. To prepare "$S_{-1}Y_D^{ox}$" and "$S_{-1}Y_D$"-thylakoids, the samples ($S_1Y_D^{ox}$- and S_1Y_D-thylakoids at a concentration of 1 mg Chl/mL) were incubated in the presence of 2 mM NH_2NH_2 for 10 min on ice at pH 7.2. It has to be emphasized that under our conditions NH_2NH_2 does not reduce the level of Y_D^{ox}. After this treatment, the samples were diluted 10-fold in buffer B [300 mM mannitol, 10 mM $MgCl_2$, 20 mM $CaCl_2$, and 50 mM Hepes (pH 7.2)], centrifuged, washed, and resuspended at a concentration of 1 mg Chl/mL. These preparations will be referred to as $S_{-1}Y_D^{ox}$- and $S_{-1}Y_D$-thylakoids, respectively. Illumination of these samples with one flash leads to a high population of the S_0 state ("$S_0Y_D^{ox}$"- and "S_0Y_D"-thylakoids). A high population of S_{-2} can be achieved by dark incubation of $S_1Y_D^{ox}$- and S_1Y_D-thylakoids for 10 min on ice in the presence of 2 mM NH_2NH_2 as described above, illumination with one flash, and then another 10 min of dark incubation in the presence of 2 mM NH_2NH_2. After this treatment, the samples were diluted, centrifuged, washed, and resuspended as in the case of $S_{-1}Y_D^{ox}$- and $S_{-1}Y_D$-thylakoids. Preparations with a high S_{-2} population will be symbolized as "$S_{-2}Y_D^{ox}$"- and "$S_{-2}Y_D$"-thylakoids.

Flash-induced O_2 oscillation patterns were measured at 20 °C with a modified Joliot-type electrode (Joliot, 1972) that keeps the temperature of the buffer reservoir and that at the electrode constant within ±0.2 °C (Messinger, 1993).

For each measurement, 10-mL samples suspended in buffer B to give a concentration of 1 mg Chl/μL were transferred rapidly to the Joliot-type electrode. Buffer B was also used as the flow buffer. The samples were always kept on the Pt electrode for 1 min before the measurements were started. The polarization current of −600 mV was switched on 30 s before excitation with a flash train (2 Hz) of short (FWHM = 3 μs) saturating Xe flashes (General Radio Stroboslave 1539A).

The amperometric signals were separated from the background signal and amplified by a laboratory-built polarograph. The amplified signal was recorded with an externally triggered Nicolet 1072. For better signal resolution, the signals were recorded with 3 ms per point starting 50 ms before and ending 120 ms after each flash. The peak heights of the signals were taken to be proportional to the amount of oxygen evolved in the corresponding flash.

The probabilities of misses (α) and double hits (β) and the apparent S_0 populations, $[S_0]_{app}$, were determined for $S_1Y_D^{ox}$-thylakoids by a least-squares fit method comparing the relative oxygen yields of the first 12 flashes of the train with theoretical calculated sequences (on the basis of the conventional Kok model) as described in Messinger et al. (1991). In order to take into account effects due to S_2 and S_3 decay and S_0 oxidation in the dark, a more elaborate Kok model was used which also permits the inclusion of additional redox states (S_{-1}, S_{-2}) (see text; U. Wacker, J. Messinger, and G. Renger, manuscript in preparation).

RESULTS

The study of interactions between Y_D^{ox} and the lower redox states, S_0, S_{-1}, and S_{-2}, of the water oxidase requires the preparation of dark-adapted samples highly enriched with a particular S_i state. Furthermore, in order to show that Y_D^{ox} is really the oxidant of the univalent redox reactions, the lifetimes of the redox states S_i should be compared in dark-adapted thylakoids containing high and low levels of Y_D^{ox}. Therefore, $S_1Y_D^{ox}$- and S_1Y_D-thylakoids (see Materials and Methods) were used as the starting material. The percentage of the reduced form, Y_D, in a particular sample can be easily determined by measuring the fast component of S_2 and/or S_3 decay. In general, the dark relaxation of S_2 and S_3 exhibits biphasic kinetics where the fast phase reflects the univalent reduction by Y_D and the slow phase reflects the reaction of S_2 and S_3 with other endogenous reductants. However, it must be emphasized that the reduction of S_2 and S_3 by Y_D is pH dependent (Vass & Styring, 1991; Renger et al., 1992). The latest findings reveal that in thylakoids the fast phase reflects the extent of reduced Y_D only at pH values of the suspension higher than about 7.0 (J. Messinger and G. Renger, manuscript in preparation). Accordingly, the experiments were performed at pH 7.2, i.e., in the plateau region of the maximum fraction of functionally fully competent water oxidase (Renger et al., 1977). The time courses of S_2 and S_3

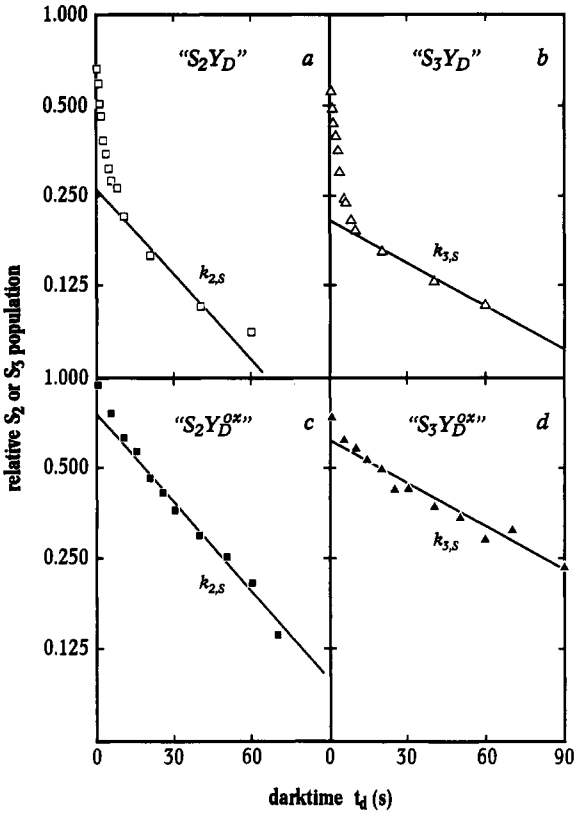

FIGURE 1: Relative S_i state populations as a function of dark time (t_d) between the 1st and 2nd flashes (S_2) or the 2nd and 3rd flashes (S_3) in S_1Y_D-thylakoids (top) or $S_1Y_D^{ox}$-thylakoids (bottom) at 20 °C and pH 7.2. The amount of Y_D was calculated from the fast phase of S_2 and S_3 decay to be 72% in S_1Y_D-thylakoids and 25% in $S_1Y_D^{ox}$-thylakoids.

determined at pH 7.2 by the conventional preflash method (Joliot & Kok, 1975) are depicted in Figure 1 for the $S_1Y_D^{ox}$- and S_1Y_D-thylakoids used in this study. The data reveal that $S_1Y_D^{ox}$-thylakoids are enriched with Y_D^{ox} (about 75% population), while S_1Y_D-thylakoids contain more than 70% of Y_D in the reduced state. For the rate constants of the fast (f) and slow (s) phases of S_2 and S_3 relaxation, the following values were obtained at 20 °C: $k_2(f) = 0.36\ s^{-1}$, $k_2(s) = 0.023\ s^{-1}$, $k_3(f) = 0.40\ s^{-1}$, and $k_3(s) = 0.014\ s^{-1}$.

The electron transfer to Y_D^{ox} from the water oxidase in redox state S_0 is known to take place within the time domain of minutes (Styring & Rutherford, 1987; Messinger et al., 1991). Accordingly, the period four oscillation of the oxygen yield patterns observed in thylakoids initially enriched in a particular redox state S_i ($i = 1, 0, -1, -2$) and subsequently incubated in the dark at 20 °C for 1 and 30 min, respectively, should differ markedly if S_i becomes oxidized by Y_D^{ox}. The flash-induced oscillation patterns obtained in $S_iY_D^{ox}$-thylakoids ($i = 1, 0, -1, -2$) are summarized in Figure 2. A comparison of these traces readily shows that a 30 min dark incubation markedly affects the patterns in $S_0Y_D^{ox}$- and $S_{-2}Y_D^{ox}$-thylakoids, while significantly less pronounced modifications take place in $S_{-1}Y_D^{ox}$-thylakoids and virtually no change is observed in $S_1Y_D^{ox}$ samples. The invariance of the oscillation pattern to a 30-min dark incubation of $S_1Y_D^{ox}$-thylakoids is expected because Y_D^{ox} cannot oxidize S_1 and Y_D^{ox} is rather stable, with lifetimes on the order of days at 0 °C (Messinger et al., 1993). Likewise, the marked change due to dark incubation of $S_0Y_D^{ox}$-thylakoids is easily understandable as the consequence of S_0 oxidation by Y_D^{ox}. Three

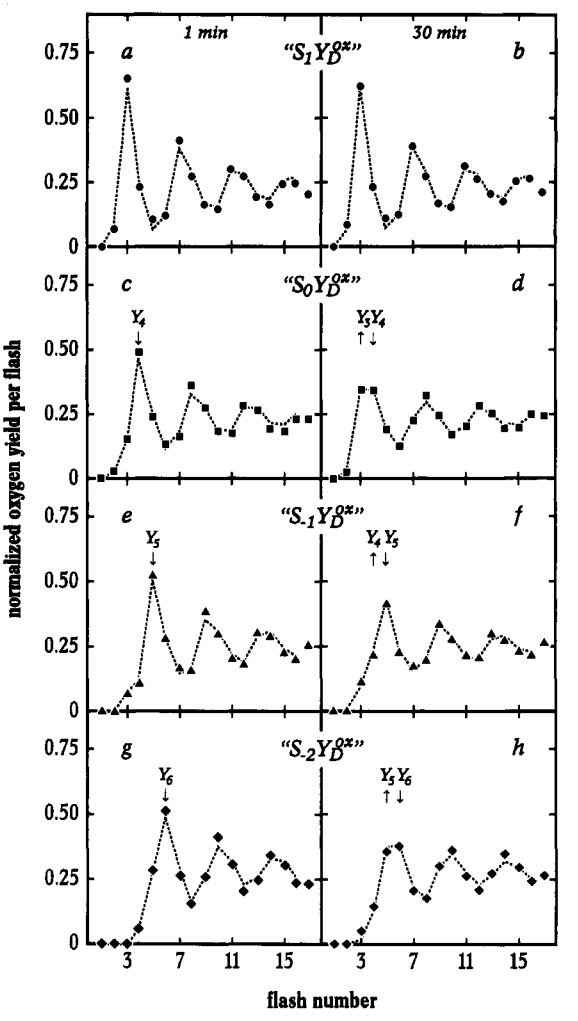

FIGURE 2: Normalized oxygen yield per flash as a function of the flash number of saturating single turnover flashes in $S_iY_D^{ox}$-thylakoids of spinach after 1 (left) and 30 min (right) of dark incubation at 20 °C and pH 7.2. The $S_iY_D^{ox}$ states were populated in the samples by NH_2NH_2 incubation and one preflash (in case of S_0 and S_{-2}) as described in Materials and Methods. The NH_2NH_2 was removed from the thylakoids before the incubation at 20 °C by washing twice with buffer. The data were normalized on the sum of the O_2 yields of flashes 3–14 (one-third of the value of this sum was set to 1). Dashed curves: Fits with the extended Kok model. The values for the S_i state populations at $t_{inc} = 0$ min are given in Table I. The arrows indicate the characteristic flash numbers of the S_i states. Further experimental details were as described in Materials and Methods.

surprising phenomena, however, are observed for S_{-1} and S_{-2}: (i) redox state S_{-1} seems to be markedly more resistant than S_0 to an oxidative attack by Y_D^{ox}; (ii) the lifetime of S_{-1} exceeds that of S_{-2}; and (iii) the population of redox states S_{-1} and/or S_{-2} does not drastically enhance the sensitivity of the water oxidase toward degradation of its functional integrity. In all samples, about 40–50% of the oxygen evolution capacity (average yield per flash) was lost during a 40-min dark incubation at 20 °C. This phenomenon is practically independent of the S_i state ($i = 1, 0, -1, -2$) and the redox state of Y_D (data not shown).

The peculiar properties of the redox states S_0, S_{-1}, and S_{-2} in terms of their reactivity with Y_D^{ox} are of interest because they could provide further information on the redox properties of the water oxidase. In order to analyze the kinetic properties in more detail, the oscillation patterns were measured as a function of dark incubation time in $S_1Y_D^{ox}$-, $S_0Y_D^{ox}$-, $S_{-1}Y_D^{ox}$- and $S_{-2}Y_D^{ox}$-thylakoids, respectively. The normalized ampli-

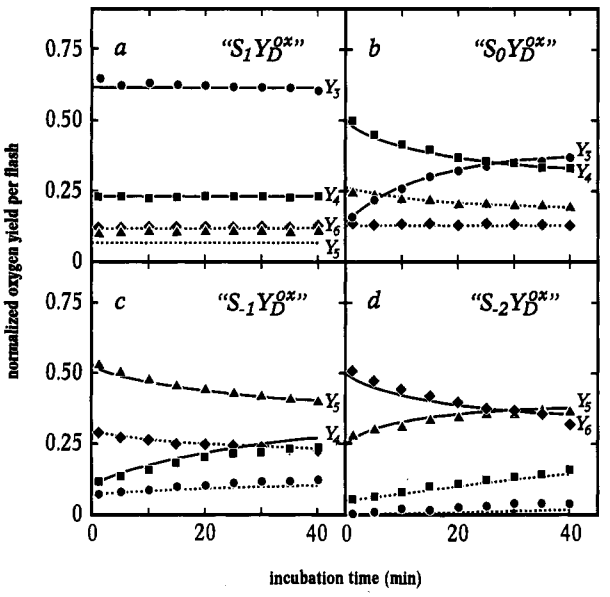

FIGURE 3: Normalized oxygen yields of flashes 3–6 (Y_3 (●), Y_4 (■), Y_5 (▲), Y_6 (◆)) as a function of dark incubation time at 20 °C and pH 7.2 in the $S_1Y_D^{ox}$-, $S_0Y_D^{ox}$-, $S_{-1}Y_D^{ox}$- and $S_{-2}Y_D^{ox}$-thylakoids of spinach. The values for Y_3, Y_4, Y_5, and Y_6 that are calculated by using the extended Kok model with the initial S_i state values and the rate constants given in Table I and Figure 1, respectively, are presented as curves (full or dotted). The full curves show the calculated time course of the oxygen yield induced by flashes which characteristically reflect the interaction of a particular S_i state (S_1, S_0, S_{-1}, S_{-2}) with Y_D^{ox}. The rate constant of this process was the free running parameter of the fit. The values obtained are given in the text. For further details see Figure 2 and the text.

tudes of the oxygen yield due to the 3rd, 4th, 5th, and 6th flashes, (symbolized by Y_3, Y_4, Y_5, and Y_6), respectively, of $S_1Y_D^{ox}$-, $S_0Y_D^{ox}$-, $S_{-1}Y_D^{ox}$- and $S_{-2}Y_D^{ox}$-thylakoids are depicted in Figure 3 as a function of the dark incubation time. It is obvious that virtually no changes take place during a 40-min dark time in $S_1Y_D^{ox}$-thylakoids. In contrast, the yields Y_3 and Y_4 in $S_0Y_D^{ox}$ - and Y_5 and Y_6 in $S_{-2}Y_D^{ox}$-thylakoids exhibit striking time dependencies of opposite sign. A similar, but less pronounced, phenomenon is observed for Y_4 and Y_5 in $S_{-1}Y_D^{ox}$-thylakoids. In order to evaluate the data quantitatively an extended Kok-model is required which takes into account (a) participation of the additional redox states S_{-2} and S_{-1} and (b) redistribution of the S_i state population during the dark incubation time before the flash sequence and the time between the flashes.

The former phenomenon can be accounted for by an extension of the S_i state population vectors and the transition matrix as described in Messinger et al. (1991). It must be emphasized that, for the sake of simplicity, the probabilities of misses (α) and double hits (β) are approximated by average values that are independent of the S_i state. Furthermore, the modulation of the α factor by acceptor side effects due to the binary oscillation of the Q_B^- state population [for a discussion, see Renger and Hanssum (1988)] will be ignored. The latter assumption can be rationalized by the fact that no special efforts were made to achieve a high population of Q_B in the dark adapted samples [see Rutherford et al. (1984)].

For the calculation of the dark redistribution of the S_i state population, the redox-active tyrosine Y_D is assumed to interact exclusively with the water oxidase of the same PS II complex. Accordingly, the whole ensemble always attains two classes of substates: $S_iY_D^{ox}$ ($i = -2, ..., +3$) and S_iY_D ($i = -2, ..., +3$). This gives rise to the following differential equations:

$$d[S_3Y_D]/dt = -(k_3(f) + k_3(s))[S_3Y_D]$$

$$d[S_2Y_D]/dt = -(k_2(f) + k_2(s))[S_2Y_D] + k_3(s)[S_3Y_D]$$

$$d[S_1Y_D]/dt = k_2(s)[S_2Y_D] + k_0[S_0Y_D^{ox}]$$

$$d[S_0Y_D]/dt = k_{-1}[S_{-1}Y_D^{ox}]$$

$$d[S_{-1}Y_D]/dt = k_{-2}1[S_{-2}1Y_D^{ox}]$$

and

$$d[S_3Y_D^{ox}]/dt = -k_3(s)[S_3Y_D^{ox}]$$

$$d[S_2Y_D^{ox}]/dt = -k_2(s)[S_2Y_D^{ox}] + k_3(s)[S_3Y_D^{ox}] + k_3(f)[S_3Y_D]$$

$$d[S_1Y_D^{ox}]/dt = k_2(s)[S_2Y_D^{ox}] + k_2(f)[S_2Y_D]$$

$$d[S_0Y_D^{ox}]/dt = -k_0[S_0Y_D^{ox}]$$

$$d[S_{-1}Y_D^{ox}]/dt = -k_{-1}[S_{-1}Y_D^{ox}]$$

$$d[S_{-2}Y_D^{ox}]/dt = -k_{-2}[S_{-2}Y_D^{ox}]$$

where $[S_{-i}Y_D^j]$ symbolizes the normalized population of a particular redox state S_i in a PS II complex with Y_D in redox state j (ox or red), $k_2(n)$ and $k_3(n)$ are the rate constants of S_2 and S_3 decay, respectively (n = fast or slow), and k_0, k_{-1}, and k_{-2} are the rate constants of Y_D^{ox}-induced univalent oxidation of S_0, S_{-1}, and S_{-2}, respectively. The rate constants of the slow S_2 and S_3 decay via reactions with endogenous electron donors other than Y_D are assumed to be independent of the redox state of Y_D.

The S_i state populations of the dark adapted thylakoids in the different states $[S_iY_D^{ox}]$ and $[S_iY_D]$ obtained from the numerical evaluation of the experimental data (see dashed lines) of Figure 2 and Figure 4 (vide infra) under the assumption of constant α ($\alpha = 0.10$) and β values ($\beta = 0.03$) are summarized in Table I. It shows that the desired high population of a particular S_i state (symbolized by a box) can be achieved by the NH_2NH_2 treatment described in this study.

As can be seen, the normalized populations of S_1 and S_{-1} in $S_1Y_D^{ox}$- and $S_{-1}Y_D^{ox}$-thylakoids, respectively, significantly exceed those of S_0 and S_{-2} in $S_0Y_D^{ox}$- and $S_{-2}Y_D^{ox}$-thylakoids. This phenomenon is mainly due to the probability of misses of the single flash which is used in order to obtain $S_0Y_D^{ox}$- and $S_{-2}Y_D^{ox}$-thylakoids.

The numerical fit of the results presented in Figure 3 leads to the following rate constants k_i ($i = -2, -1, 0$) for the univalent oxidation by Y_D^{ox} of S_{-2}, S_{-1}, and S_0, respectively: $k_0 = 9 \times 10^{-4}\ s^{-1}$, $k_{-1} = 4 \times 10^{-4}\ s^{-1}$, and $k_{-2} = 9 \times 10^{-4}\ s^{-1}$. These rate constants clearly show that S_{-2} and S_0 undergo univalent oxidation with virtually identical kinetics while S_{-1} exhibits a slower electron abstraction. The kinetic analysis relies on the assumption that Y_D^{ox} acts as the oxidant. In order to check this idea, the corresponding experiments were performed in S_iY_D-thylakoids which retain only a minor fraction of Y_D^{ox} (about 25%). In this case, the flash-induced oscillation patterns of the oxygen yield should be much less affected by a dark incubation of 30 min. The results obtained are summarized in Figure 4. As expected, no significant changes are observed for S_iY_D-thylakoids enriched in any particular S_i state (i = –2, –1, 0, and 1). This phenomenon markedly contrasts with the observations in $S_iY_D^{ox}$-thylakoids (see Figure 2). In order to illustrate the kinetic properties of the redox states below S_1 in thylakoids with a high dark population

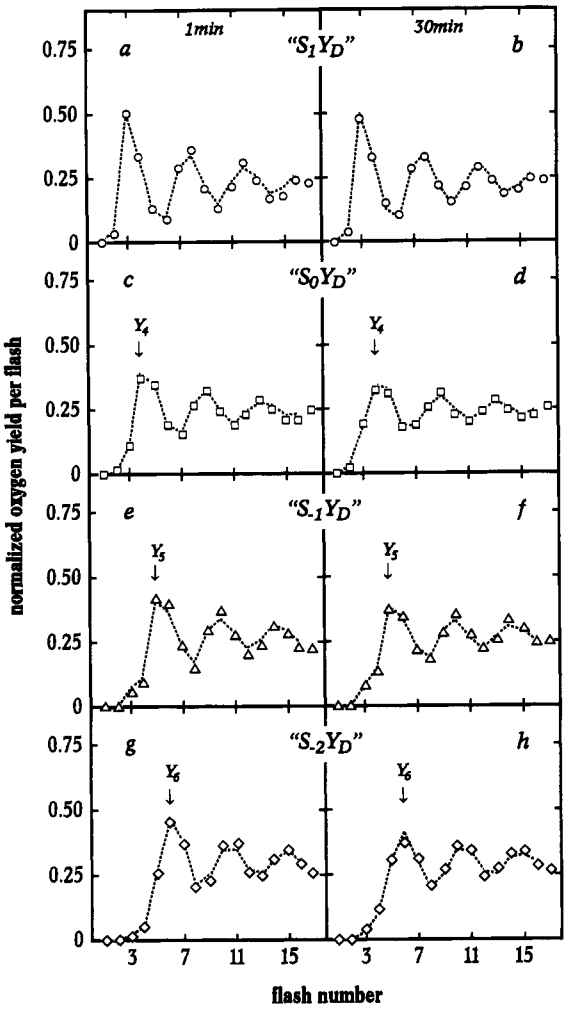

FIGURE 4: Normalized oxygen yield per flash as a function of the flash number of saturating single turnover flashes in S_iY_D-thylakoids of spinach after 1 (left) and 30 min (right) of dark incubation at 20 °C and pH 7.2. For further details, see Figure 2 and the text. Dashed curves: Fits with the extended Kok model. The values for the S_i state populations at t_{inc} = 0 min are given in Table I.

of reduced Y_D, the oxygen yields due to flashes 3–6 were measured as a function of dark incubation time. The results obtained are summarized in Figure 5. The curves in Figure 4 (dashed) and Figure 5 (full and dashed) represent numerical fits with the same rate constants used in Figures 2 and 3 and the normalized S_i state populations given in Table I. A comparison with Figure 3 readily shows that only a marginal dark redistribution of the S_i state populations takes place in S_iY_D-thylakoids. This finding strongly supports the idea that Y_D^{ox} is a unique oxidant in the PS II complex for univalent electron abstraction from the water oxidase in redox states S_{-2}, S_{-1}, and S_0.

A last interesting point should be addressed briefly. The S_0 state in the S_0Y_D- and $S_0Y_D^{ox}$-thylakoids was generated by using an NH_2NH_2-induced transformation into S_{-1} and subsequent flash oxidation to S_0 by excitation with a saturating flash. Therefore, the question arises as to whether or not this S_0 state exhibits the same redox interaction kinetics with Y_D^{ox} as the S_0 state populated through a three-preflash oxidative cycle via S_4 and O_2 evolution of the water oxidase. In this case, a population of about 65% S_0 can be achieved. At definite dark times after the preflash treatment, the oscillation patterns of oxygen evolution were determined in the same way as that described for the NH_2NH_2-treated

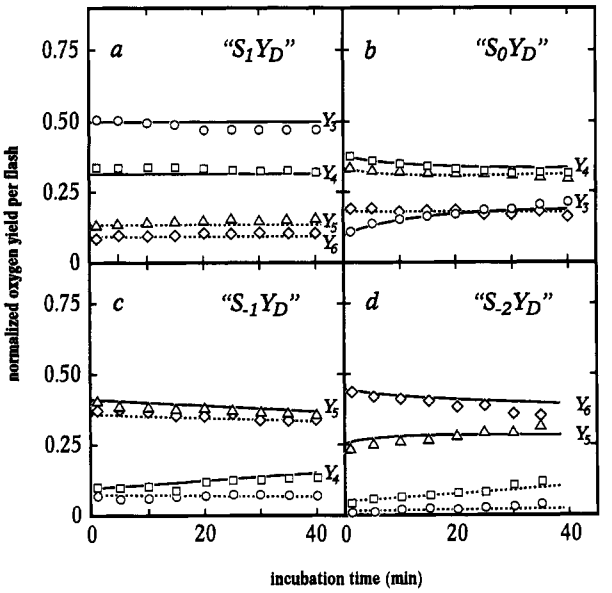

FIGURE 5: Normalized oxygen yields of flashes 3–6 (Y_3 (○), Y_4 (□), Y_5 (△), Y_6 (◇)) as a function of dark incubation time at 20 °C and pH 7.2 in S_1Y_D-, S_0Y_D-, $S_{-1}Y_D$-, and $S_{-2}Y_D$-thylakoids of spinach. The values for Y_3, Y_4, Y_5, and Y_6 that are calculated by using the kinetic S_i state model with the initial S_i state values and the rate constants given in Table I and Figure 1, respectively, are presented as curves (full or dotted). The full curves show the calculated time course of the oxygen yield induced by flashes which characteristically reflect the interaction of a particular S_i state (S_1, S_0, S_{-1}, S_{-2}) with Y_D^{ox}. The same rate constants were used as in Figure 3.

Table I: Initial (time = 0) Normalized S_i State Population, [S_i], in $S_iY_D^{ox}$- and S_iY_D-Thylakoids[a]

thylakoids	[S_{-2}]	[S_{-1}]	[S_0]	[S_1]	[S_2]
$S_1Y_D^{ox}$	0	0	0	1.00	0
S_1Y_D	0	0	0.10	0.90	0
$S_0Y_D^{ox}$	0	0.08	0.77	0.02	0.13
S_0Y_D	0.01	0.24	0.65	0.01	0.10
$S_{-1}Y_D^{ox}$	0	0.90	0	0.10	0
$S_{-1}Y_D$	0.16	0.74	0	0.10	0
$S_{-2}Y_D^{ox}$	0.72	0.28	0	0	0
$S_{-2}Y_D$	0.64	0.32	0.02	0.02	0

[a] The symbols $S_iY_D^{ox}$ and S_iY_D refer to samples of thylakoids with high Y_D^{ox} (and low Y_D) and high Y_D (and low Y_D^{ox}) levels, respectively, which were enriched in a definite S_i state ($i = 0, -1, -2$) by a suitable pretreatment as described in Materials and Methods. The normalized S_i populations of these samples were calculated by fitting, with the extended Kok model, the measured oscillation patterns obtained for each of the pretreated thylakoids (see text).

samples. The data obtained for the oxygen yields Y_3–Y_6 as a function of dark incubation time closely resemble those of Figure 3 for $S_0Y_D^{ox}$-thylakoids (not shown). A numerical evaluation of these results leads to a rate constant for S_0-oxidation of $k_0 = 8.5 \times 10^4\ s^{-1}$. Within an experimental error of about 10%, this value is identical with that measured in $S_0Y_D^{ox}$-thylakoids where S_0 is generated via an NH_2NH_2 preflash procedure. This observation provides strong evidence for the assumption that both S_0 states generated by different procedures exhibit the same electronic configuration.

DISCUSSION

The results of this study reveal that NH_2NH_2 is a suitable reductant for generating comparatively high population probabilities of the redox states S_0, S_{-1}, and S_{-2} in the water oxidase of isolated spinach thylakoids. These S_i states are

more reduced than S_1, which was found to be the most stable redox state of the water oxidase (Vermaas et al., 1984) in a dark-relaxed normal PS II complex at room temperature. It is shown that S_0, S_{-1}, and S_{-2} slowly donate an electron to the oxidized form (Y_D^{ox}) of the redox-active tyrosine in polypeptide D2. Furthermore, Y_D^{ox} seems to be a unique oxidant of the PS II complex because in S_iY_D-thylakoids only marginal oxidation of the redox states S_i ($i = 0, -1, -2$) is observed within a 40-min time domain. The low level of Y_D^{ox} in these samples can fully account for this small extent of slow S_i state oxidation. Three striking phenomena emerge from the kinetic analysis of the results within the framework of an "extended" Kok model: (i) the kinetics of S_{-2} and S_0 oxidation with Y_D^{ox} are virtually identical; (ii) S_{-1} exhibits a significantly slower electron transfer to Y_D^{ox}; and (iii) both S_0 states generated from S_1, either through a flash-induced, three-step oxidative pathway of the water oxidase with subsequent reduction under O_2 formation or through reductive NH_2NH_2 treatment including a one-flash-induced oxidation step, are characterized by the same kinetics of oxidation by Y_D^{ox}. On the basis of these results, and taking into account recent findings reported in the literature, an attempt will be made to assign the redox states S_{-1} and S_{-2} to redox groups of the PS II complex.

It was previously assumed that NH_2NH_2 and NH_2OH are able to reduce a two electron redox component C which directly interferes with the univalent oxidation steps of the water oxidase. The endogenous species C either could be a (binuclear) manganese group not directly undergoing redox transitions during the oxidative pathway $S_i \rightarrow S_{i+1}$ ($i = 0, ..., 3$) or it could be a non-manganese group [for a detailed discussion, see Messinger et al. (1991)]. The latest structural studies led to the interpretation of the tetranuclear manganese cluster of the water oxidase as a dimer of two binuclear centers (Yachandra et al., 1992; Kusunoki, 1992a,b; Zheng & Dismukes, 1992). It therefore appears very attractive to correlate this structural feature with a functional heterogeneity of the four manganese centers. Accordingly, in line with previous postulates (Renger, 1978, 1987b), one binuclear manganese group, referred to as $[Mn(n)–Mn(m)]_{wox}$ (n and m symbolize the oxidation states of the two manganes atoms in a particular redox state S_i with $i \geq 0$), forms the catalytic core of the water oxidase (wox) which (together with its ligand sphere including substrate water) directly participates in the normal Kok cycle. The other binuclear manganese center could act as component C, which is reducable by exogenous substances such as NH_2NH_2. This assignment of component C to a binuclear manganese group seemed to be at variance with previous conclusions gathered from XANES (X-ray absorption near edge structure) measurements, where manganese was claimed to remain unaffected in its redox state by dark incubation of PS II membrane fragments with NH_2OH leading to S_{-1} (Guiles et al., 1990). The latest findings, however, revealed that dark treatment of PS II core complexes with exogenous reductants such as hydroquinone or NH_2OH really causes manganese reduction (Riggs et al., 1992).

The characterization of the different S_i states within the framework of the heterogeneous model with two different binuclear manganese groups requires detailed information on the nature of at least one particular redox state of the water oxidase.

There is now convincing evidence that the tetranuclear manganese cluster is characterized by the electronic configurations $Mn(III)_2–Mn(IV)_2$ and $Mn(III)–Mn(IV)_3$ in redox states S_1 and S_2, respectively [see Kusunoki (1992a,b) and references therein]. Furthermore, in most schemes currently discussed, two of the four manganese atoms in the tetranuclear cluster are assumed to remain redox-inactive during the oxidative pathway from S_0 to S_3 [for recent reviews, see Rutherford et al. (1992) and Debus (1992)]. On the basis of this idea, the electronic configurations of the manganese atoms in the catalytic binuclear center are well established as to be $[Mn(III)–Mn(III)]_{wox}$ in S_1 and $[Mn(III)–Mn(IV)]_{wox}$ in S_2. Therefore, the remaining binuclear Mn(IV)–Mn(IV) group could be identified as component C. It will be symbolized by $[Mn(IV)–Mn(IV)]_C$. The thermodynamic properties of redox transitions within this binuclear group $[Mn(IV)–Mn(IV)]_C$ are assumed to differ markedly from those of the catalytic binuclear group, $[Mn(n)–Mn(m)]_{wox}$, due an asymmetric protein environment around the tetranuclear cluster as is shown schematically in Figure 6 and discussed extensively in a previous report (Renger, 1987b).

On the basis of the above mentioned considerations, and taking into account the electronic configurations of S_1 and S_2 (vide supra), the nature of redox states S_0, S_{-1}, and S_{-2} can be discussed. As originally proposed by Andreasson et al. (1983), the $S_0 \rightarrow S_1$ transition probably comprises an Mn(II) oxidation to Mn(III). This assignment, which is in line with previous X-ray absorption results (Guiles et al., 1990; Ono et al., 1992), implies that the oxidation potential of this Mn(II) center within $[Mn(II)–Mn(III)]_{wox}$ is below that of Y_D in order to permit electron abstraction from S_0 by Y_D^{ox}. If one accepts that the superreduced states are metal-centered, two electronic configurations can be considered for S_{-2}: $\{[Mn(II)–M(III)]_{wox}[Mn(III)–Mn(III)]_C\}$ and $\{[Mn(II)–Mn(II)]_{wox}[Mn(III)–Mn(IV)]_C\}$. Although, a priori, no unambiguous assignment can be made, the former configuration seems to be more attractive. It provides a simple explanation for the virtually identical reoxidation kinetics of S_0 and S_{-2} by Y_D^{ox} because the same redox state of the catalytic site would be involved. This argument, however, is only tenable if the redox state of component C does not affect the electron-transfer kinetics from $[Mn(II)–Mn(III)]_{wox}$ to Y_D^{ox}. Within the framework of the above assignment, S_{-2} would correspond with $[S_0]_{wox}C_{2red}$, where C_{2red} is the double reduced form of component C, i.e., $C_{2red} = [Mn(III)–Mn(III)]_C$.

A more complex situation arises for redox state S_{-1}. Following the hypothesis of functional heterogeneity with two binuclear manganese groups of different redox properties, three electronic configurations have to be taken into consideration for S_{-1}: $\{[Mn(II)–Mn(III)]_{wox}[Mn(III)–Mn(IV)]_C\}$, $\{[Mn(II)–Mn(II)]_{wox}[Mn(IV)–Mn(IV)]_C\}$, and $\{[Mn(III)–Mn(III)]_{wox}[Mn(III)–Mn(III)]_C\}$. These states can be described formally by $[S_0]_{wox}C_{1red}$, $[S_{-1}]_{wox}C$, and $[S_1]_{wox}C_{2red}$, respectively. For the sake of a uniform reaction pattern, the configuration $[S_1]_{wox}C_{2red}$ would be most attractive because in this case the NH_2NH_2 induced formation of S_{-1} and S_{-2} from S_1 and S_0, respectively, would comprise the same two-electron redox reaction with unit C, i.e., $C \xrightarrow{NH_2NH_2} C_{2red}$.

This idea is supported by our recent finding that the NH_2NH_2-mediated population of S_{-1} and S_{-2} strongly depends on the S_1/ S_0 ratio of the dark-adapted thylakoids before treatment with the reductant (Renger et al., 1990). The proposed configuration $[S_1]_{wox}C_{2red}$ of S_{-1} implies that Y_D^{ox} should oxidize the double-reduced binuclear manganese group, C_{2red}, rather than the catalytic unit $[...]_{wox}$ because the reaction $[Mn(III)–Mn(III)]_{wox}(Y_D^{ox}) \rightarrow [Mn(III)–Mn(IV)]_{wox}(Y_D)$ in redox state S_1 and therefore also in S_{-1} is prevented for thermodynamic reasons (Vass & Styring, 1991), provided

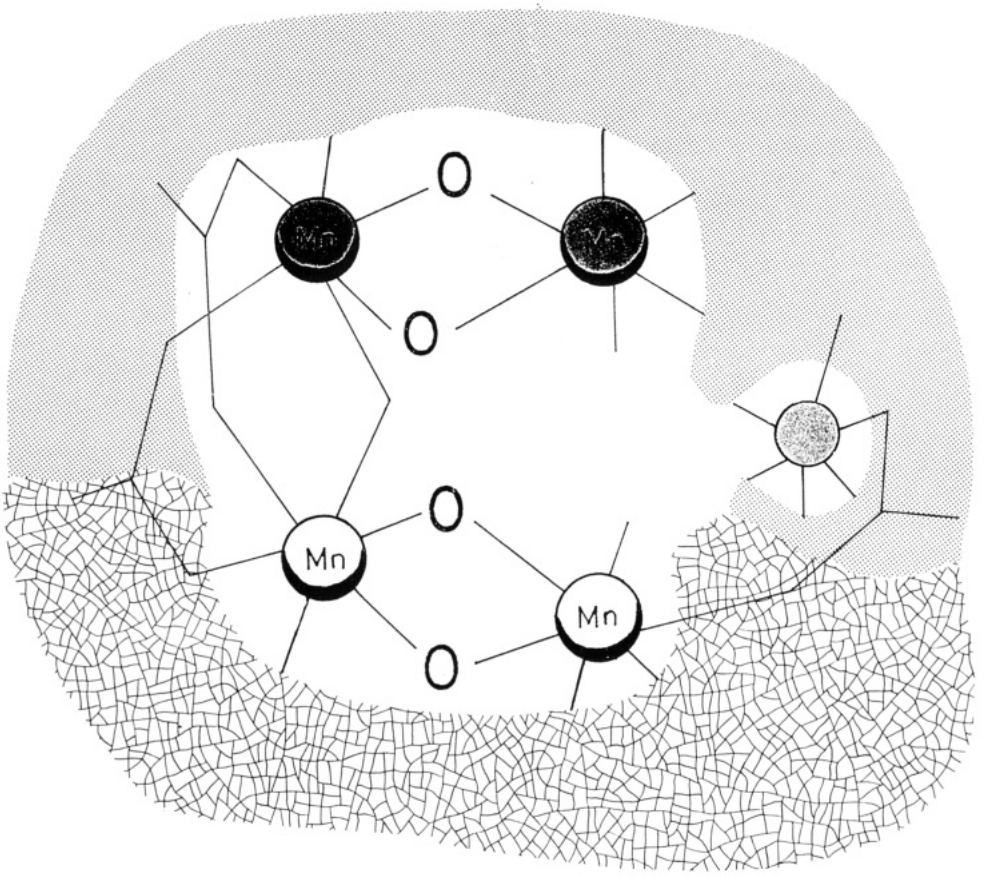

FIGURE 6: Schematic representation of the tetranuclear cluster of the water oxidase consisting of two binuclear μ-oxo-bridged manganese groups in an asymmetric protein matrix. The arrangement of the manganes atoms and their bridging ligands corresponds with a recent proposal of Kim et al. (1991), but it must be emphasized that other models have been discussed where the bridging between the manganes atoms of the binuclear groups is different [for discussion, see Kusunoki et al. (1992a,b)]. The asymmetry of the protein environment is symbolized by differently hatched areas. The upper μ-oxo-bridged group is assumed to be the catalytic site represented in the text by $[Mn(n)–Mn(m)]_{wox}$, and the bottom group is proposed to be component C, i.e., $[Mn(IV)–Mn(IV)]_C$. The grey circle symbolizes a Ca^{2+} ion which is assumed to play an important role for the function of the water oxidase.

that the redox state of the dimeric unit C does not markedly affect the energetics of Mn(III) oxidation in the catalytic unit $[...]_{wox}$. Therefore, the assignment of S_{-1} to $[S_1]_{wox}C_{2red}$ constitutes a different oxidative pathway of S_{-1} and S_{-2} with Y_D^{ox}, i.e., univalent C_{2red} versus $[S_0]_{wox}$ oxidation, respectively. This would simply explain the differences in the kinetics. However, it must be emphasized that a slight distance difference due to structural changes of the water oxidase could be also responsible for the kinetic effects. The oxidation of configuration $[S_1]_{wox}C_{2red}$ by Y_D^{ox} could create a formal S_0 state with an electronic configuration different from normal S_0. Although information is lacking, it seems reasonable to speculate that the electron abstraction from C is followed by an electron transfer between both dimeric units in the tetranuclear cluster: $\{[Mn(III)–Mn(III)]_{wox}[Mn(III)–Mn(IV)]_C\} \rightarrow \{[Mn(II)–Mn(III)]_{wox}[Mn(IV)–Mn(IV)]_C\}$. This reaction gives rise to the conventional electronic configuration of S_0. In this way, the oxidation of S_{-1} by Y_D^{ox} can be interpreted as an oxidant-induced reduction of the catalytic binuclear manganese unit: $[S_1]_{wox}C_{2red}Y_D^{ox} \rightarrow [S_1]_{wox}C_{1red}$-$Y_D \rightarrow [S_0]_{wox}CY_D$.

Recently, indirect evidence has been presented for other possible electronic configurations of S_{-1}. It was concluded that the reduction of salt-treated PS II membrane fragments with NH_2OH leads to an S_{-1} state with $Mn(II)–Mn(III)_2–Mn(IV)$, while hydroquinone gives rise to an S_{-1} state with $Mn(II)_2–Mn(IV)_2$ (Mei & Yocum, 1992). These states would correspond with $[S_0]_{wox}C_{1red}$ and $[S_{-1}]_{wox}C$, respectively. On the other hand, it has been reported that XANES data obtained in PS II core complexes reduced with NH_2OH can be interpreted either by $[Mn(III)]_4$, i.e., corresponding with $[S_1]_{wox}C_{2red}$, or by the presence of one Mn(II) center. In the case of hydroquinone, one or two manganese atoms can attain the redox state Mn(II) (Riggs et al., 1992). A closer inspection of the data, however, reveals that the possibility of S_{-2} formation is not entirely excluded [this state necessarily implies the presence of Mn(II)]. Therefore, a straightforward conclusion cannot be achieved at this stage of knowledge.

Regardless of these details, it must be emphasized that the actual redox state of each manganese atom within the tetranuclear cluster at a definite S_i state of the water oxidase strongly depends on the microenvironment. Therefore, varying electronic distributions among the metal centers can arise in differently treated samples, especially after the removal of extrinsic regulatory subunits.

So far, the redox properties of the manganese centers in the tetranuclear cluster and their possible modulation by an asymmetric protein environment have been discussed. These points are certainly of central relevance for the interactions with endogenous redox components. If one takes into account the reaction with substrate water and specific modifications by exogenous redox active substances, the possibility of different accessibilities of the dimeric manganese units $[...]_{wox}$ and $[...]_C$ also have to be considered. At present, no information is available on this point. The latest results provide evidence of an important regulatory role for the extrinsic membrane-bound subunits as shield of the water oxidase to exogenous water-soluble reductants [see Mei and Yocum

(1992) and references therein]. Likewise, structural changes of the water oxidase coupled with the $S_2 \rightarrow S_3$ transition are assumed to be responsible for the markedly slower reduction of S_3 (compared with S_2) by NH_2NH_2 and NH_2OH (Messinger & Renger, 1990; Messinger et al., 1991). Interestingly, the kinetics of S_2 and S_3 reduction by the endogenous electron donor Y_D are very similar above pH 6.5, but exhibit marked differences between both redox states in the acidic region (Messinger & Renger, 1992). The implications of this phenomenon will be addressed in a forthcoming paper.

The results of the present study raise questions about the relevance of the redox states S_{-1} and S_{-2}. There are reports in the literature which indicate that S_{-1} and S_{-2} are not only "exotic" states of the water oxidase created by artificial treatments with suitable reductants, such as NH_2NH_2, but that these states can be also populated in vivo (Bader et al., 1983; Shiraiwa & Schmid, 1986). Likewise, the proposed endogenous component C as a reductant of S_2 and S_3 (Lavorel & Maison-Peteri, 1983) could actually be the binuclear group C in the double-reduced form, $[Mn(III)–Mn(III)]_C$. At present, no information is available on the physiological relevance of redox states S_{-1} and S_{-2}. Further experiments are required to clarify this interesting point.

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