Volume 277, number 1,2, 141-146 FEBS 09275 December 1990

The reactivity of hydrazine with photosystem II strongly depends on the redox state of the water oxidizing system

J. Messinger and G. Renger

Max Volmer Institut für Biophysikalische und Physikalische Chemie der Technischen Universität, Straße des 17. Juni 135, D 1000 Berlin, Germany

Received 4 September 1990; revised version received 30 October 1990

The decay kinetics of the redox states S_2 and S_3 of the water-oxidizing enzyme have been analyzed in isolated spinach thylakoids in the absence and presence of the exogenous reductant hydrazine. In control samples without NH_2NH_2 a biphasic decay is observed. The rapid decline of S_2 and S_3 with Y_D as reductant exhibits practically the same kinetics with $t_{1/2}=6$–7 s at pH = 7.2 and 7°C. The slow reduction (order of 5–10 min at 7°C) of S_2 and S_3 with endogenous electron donors other than Y_D is about twice as fast for S_2 as for S_3 under these conditions. In contrast, the hydrazine-induced reductive shifts of the formal redox states S_i ($i=0...3$) are characterized by a totally different kinetic pattern: (a) at 1 mM NH_2NH_2 and incubation on ice the decay of S_2 is estimated to be at least 25 times faster ($t_{1/2}<0.4$ min) than the corresponding reaction of S_3 ($t_{1/2}\approx 13$ min); (b) the NH_2NH_2-induced decay of S_3 is even slower (about twice) than the transformation of S_1 into the formal redox state 'S_{-1}' ($t_{1/2}\approx 6$ min), which gives rise to the two-digit phase shift of the oxygen-yield pattern induced by a flash train in dark adapted thylakoids. (c) the NH_2NH_2-induced transformation $S_0 \rightarrow$ 'S_{-2}' [Renger, Messinger and Hanssum (1990) in: Curr. Res. Photosynth. (Baltscheffsky, M., ed), Vol. 1, pp. 845–848, Kluwer, Dordrecht] is about three times faster ($t_{1/2}\approx 2$ min) than the reaction $S_1 \xrightarrow{NH_2NH_2}$ 'S_{-1}'. Based on these results, the following dependence on the redox state S_i of the reactivity towards NH_2NH_2 is obtained: $S_3 < S_1 < S_0 \ll S_2$. The implications of this surprising order of reactivity are discussed.

Photosystem II; Water oxidation; S_i-state lifetimes; Hydrazine; Hydroxylamine

1. INTRODUCTION

Photosynthetic water oxidation to dioxygen and H^+ release into the thylakoid lumen take place via a sequence of 4 univalent oxidation steps, comprising redox transitions at a manganese-containing unit (for recent review, see refs. 1-4). $P680^+$ acts as primary oxidant, and component Y_Z, recently identified [5] as Tyr-161 of polypeptide D_1 in *Synechocystis* sp. PCC 6803, functions as intermediate redox carrier. The nature of the manganese-containing storage unit and the catalytic site of water oxidation, however, are still unresolved problems. It is well known that the intermediary redox states referred to as S_i states [6] exhibit characteristic kinetics and thermodynamic properties but the electronic configuration and nuclear geometry of these states are unknown. Surprisingly, in dark-adapted samples, the redox state S_1 is practically exclusively populated [7]. S_2 and S_3 decay in the range of seconds up to a few minutes by electron transfer either from the reduced form of component Y_D, identified as Tyr-160 of polypeptide D_2 in *Synechocystis* sp. PCC 6803 [8,9] ('inactive branch'), or from the acceptor side quinones [10,11]. These reactions exhibit characteristic temperature dependences [12,13]. In contrast to the reduction of S_2 and S_3, S_0 becomes slowly oxidized (tens of minutes) to S_1 by the oxidized form Y_D^{ox} [14]. The lifetimes of S_2 and S_3 can be selectively modified by ADRY agents [15] and reductants like NH_2OH [16] and NH_2NH_2 [17]. The latter compounds introduce, under properly selected conditions, additional reduction equivalents which give rise to a two-flash delay induced by a flash train [18]. It was recently shown that the oxidation kinetics of S_2 and S_3 exhibit markedly different activation energies and entropies [19]. This might reflect structural differences of S_2 and S_3. Conformational changes of the water oxidizing enzyme could also affect its accessibility to exogenous redox substances. Therefore, in this communication the reactivity of the water-oxidizing enzyme system towards NH_2NH_2 was analyzed as a function of the redox state S_i. The data obtained reveal that the NH_2NH_2-induced decay of S_2 is much faster than that of S_3 while the reactions of S_2 and S_3 with endogenous reductants do not exhibit such a pronounced kinetic difference. The mechanistic implications of these findings will be discussed.

Correspondence address: G. Renger, Max Volmer Institut, Technische Universität, PC 14, Straße des 17. Juni 135, D 1000 Berlin 12, Germany

2. MATERIALS AND METHODS

Thylakoids were prepared from market spinach according to the procedure described in ref. [20]. The redox state of Y_D was properly manipulated in the following way: (A) storage in the dark at −80°C

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

led to a very slow reduction of Y_D^{ox} by electron donors other than the S_0 state of the water-oxidizing system [21], without affecting the oxygen-evolution capacity. In our samples the half time of the very slow Y_D formation was of the order of 25 weeks. The extensively dark-adapted samples, referred to as 'Y_DS_1'-thylakoids, were especially suitable for studying the kinetics of the comparatively fast univalent S_2 and S_3 reduction by Y_D. (B) Illumination of thylakoids kept on ice with one single turnover flash and subsequent incubation in the dark for 1 h leads to a population of the redox state $Y_D^{ox}S_1$ in the vast majority of PS II. Therefore, in these samples, referred to as '$Y_D^{ox}S_1$'-thylakoids, the fast S_2 and S_3 decay is largely eliminated.

The flash-induced O_2 oscillation patterns were measured with a modified Joliot-type electrode [22] that keeps the temperature of the buffer reservoir and the electrode constant within ±0.3°C. The measurements of this study were performed at an electrode temperature of 7°C. Our Joliot-type electrode does not allow rapid injection and mixing of the sample with exogenous substances. Therefore, in addition to the conventional method, the following procedure was used for the lifetime measurements: 80 μl of the sample containing '$Y_D^{ox}S_1$'-thylakoids, 0.3 M mannitol, 20 mM $CaCl_2$, 10 mM $MgCl_2$ and 50 mM Hepes/NaOH, pH 7.2, were illuminated with one or two flashes in order to populate the redox states $Y_D^{ox}S_2$ and $Y_D^{ox}S_3$, respectively. Immediately after the flash(es), 20 μl buffer (see above) is added leading to a final chlorophyll concentration of 1 mg/ml. The samples were kept on ice for the desired dark time. After this treatment, 10 μl of the suspension were rapidly transferred in the dark to the Joliot-type electrode, and the polarization (−750 mV) was switched on about 20 s before the measurement. The time required for this manipulation was about 1 min so that the total dark incubation of the state $Y_D^{ox}S_i$ (i = 2, 3) is given by the storage time in the ice bath plus the time gap of 1 min between sample transfer and measurement.

For the analysis of the NH_2NH_2 (NH_2OH) effects the same procedure was used with the exception that the added buffer contained NH_2NH_2 (NH_2OH) to achieve a final concentration of 1 mM NH_2NH_2 (50 μM NH_2OH).

3. RESULTS

The reduction of S_2 and S_3 can be determined from plots of the oxygen yield due to the 3rd flash measured as a function of the time between the 1st and 2nd flash (S_2 decay) or between the 2nd and 3rd flash (S_3 decay) [23]. In order to reduce the probabilities of misses and double hits and to increase the stability of the samples as well as the lifetime of S_2 and S_3 the measurements were performed at low temperature. As observed previously [7], dark-adapted samples exhibit a biphasic decay (see insert of Fig. 1, top, right side). The fast phase reflects the reaction of S_2/S_3 with Y_D, the slow phase the reaction with other endogenous electron donors. After one preflash the rapid decay is for the most part eliminated. The semilogarithmic plot of the data in Fig. 1 reveals that in our samples virtually no kinetic differences exist between S_2 and S_3 with regard to the electron transfer from Y_D (the half lifetimes are 7 s and 6 s, respectively, at 7°C and pH 7.2), while S_2 is reduced faster than S_3 with other endogenous donors of PS II (the slow kinetics exhibit half lifetimes of 4 and 10 min for S_2 and S_3, respectively at pH 7.2 and 7°C). If the samples are given a preflash and subsequent dark adaptation, Y_D gets oxidized by S_2 and stays oxidized in the time domain of hours due to the lack of S_0. (Interestingly, in the presence of chaotropic anions of the Hofmeister series (J^-, SCN^-, ClO_4^-) the stability of Y_D^{ox} is markedly diminished and Y_D^{ox} becomes reduced

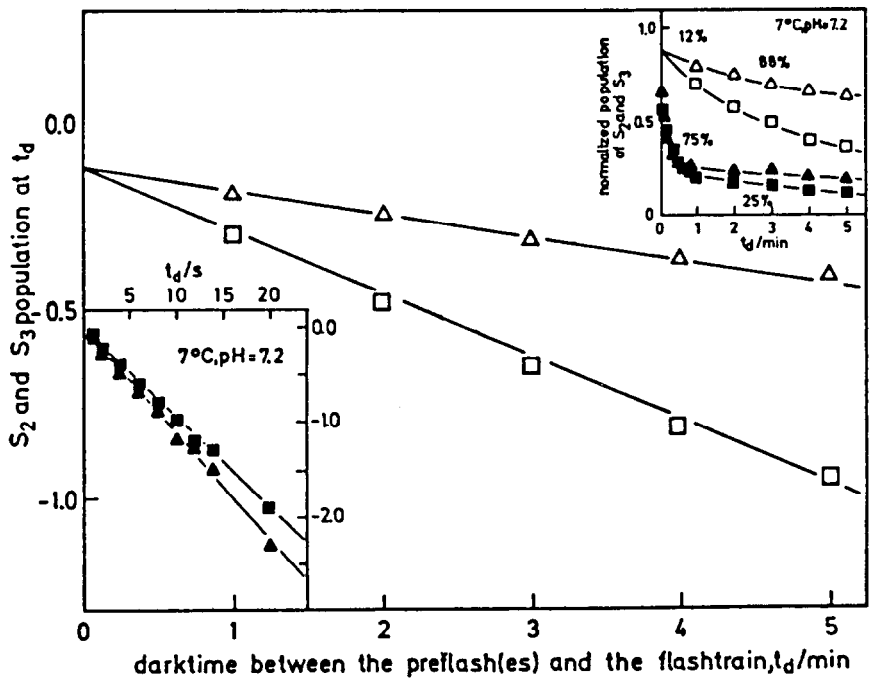

Fig. 1. Semi-logarithmic plot (ln scale) of relative S_2 (squares) and S_3 (triangles) populations as a function of dark time, t_d, between one (S_2) or two (S_3) pre-flashes and the monitoring flash train in '$Y_D^{ox}S_1$' thylakoids at δ = 7°C, pH 7.2. Insert, top right: normalized S_2 and S_3 populations as a function of dark time between pre-flashes and monitoring flash train in samples without additional flash (closed symbols) or with one additional flash given 1 h before the measurements (open symbols). Insert, bottom left: semi-logarithmic plot (ln scale) of the fast kinetics of S_2 (□) and S_3 (△) decay separated from the measurements of insert, top right. For experimental detail see section 2.

much faster in the absence of S_0 as will be outlined elsewhere (Messinger and Renger, in preparation)). Consequently, after 1 h dark adaptation on ice, practically all PS II centers are in the state $Y_D^{ox}S_1$, and electron transfer from Y_D to S_2/S_3 in the time domain of a few seconds is prevented. In this case, S_2 and S_3 become reduced in the range of minutes by electron donors other than Y_D. Although the role of Q_B^- (via Q_A^-) as reductant of S_2 and S_3 is well established (for a discussion see ref. [11]), the electron donor(s) in the absence of Y_D and semiquinones at the PS II acceptor side is (are) not yet clarified. Our first attempts failed to resolve a biphasic S_2 and S_3 decay under conditions where only part of the PS II centers containing Q_B^- and Y_D^{ox} stays oxidized (further studies are under way to address this problem). As this mechanistic detail does not affect the central conclusions of the present study, the donor components for the slow S_2 and S_3 relaxation will not be further discussed here. The elimination of the fast S_2/S_3 decay is indicated in Fig. 1 (top, right side) by the open symbols (a small remaining fast decay of about 10% is caused mainly by misses due to incomplete $S_1 \rightarrow S_2$ transition by the pre-flash).

The determination of the reactivity of S_2 and S_3 with NH_2NH_2 (and NH_2OH) cannot be directly performed at the Joliot-type electrode because the system does not permit rapid injection and mixing of the sample with external chemicals. Therefore, the procedure described in Materials and Methods was used. In the '$Y_D^{ox}S_3$' sample the oxygen yield due to the first flash of the flash train, Y_1, as a function of the total time between NH_2NH_2 addition and the measurement reflects the decay of S_3. Analogously, in the '$Y_D^{ox}S_2$' sample, the oxygen yield Y_2 indicates the S_2 decay induced by NH_2NH_2. In order to account for the slight decrease of the number of PS II centers fully competent in O_2 evolution in the presence of NH_2NH_2 (NH_2OH) under our experimental conditions, the oxygen yields Y_1 and Y_2 were normalized to the steady-state value of each measurement. The results obtained are depicted in Fig. 2. For technical reasons (see section 2), no data points could be measured within the first minute after NH_2NH_2 addition. In the presence of NH_2NH_2, the decay of S_2 and S_3 are markedly faster than in the control. However, more interesingly, the NH_2NH_2-induced decay kinetics exhibited significant differences between S_2 and S_3, in contrast to what is observed in control thylakoids. The NH_2NH_2-induced decline of S_2 exhibits overall kinetics that cannot be resolved due to the limited time resolution of our approach. Regardless of this problem, it is clear that the NH_2NH_2-induced S_2 decay is faster by more than one order of magnitude than that of S_3. Similar differences between S_2 and S_3 were also observed with NH_2OH (data not shown). In order to resolve these differences, it is indispensable to oxidize Y_D by a preflash in order to avoid interference with the fast reactions $Y_DS_i \rightarrow Y_D^{ox}S_{i-1}$ ($i = 2, 3$). This effect significantly contributed to the results of our

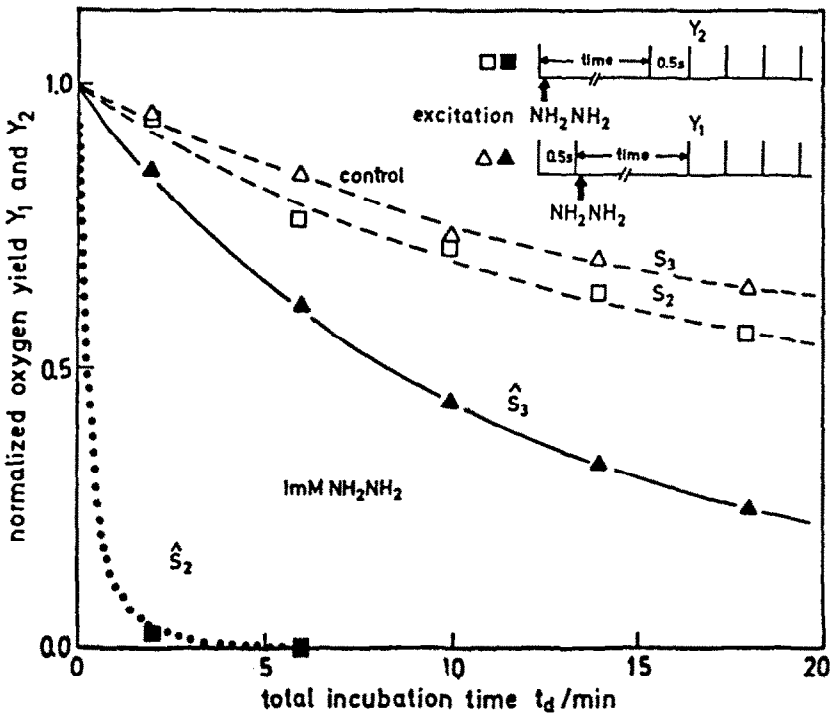

Fig. 2. Normalized oxygen yield Y_1 and Y_2 as a function of dark time between one or two pre-flashes and the detecting flash train at pH 7.2. Open symbols: control (without NH_2NH_2 addition). Closed symbols: addition of NH_2NH_2 (final concentration, 1 mM) immediately after the pre-flash(es). For experimental details see section 2.

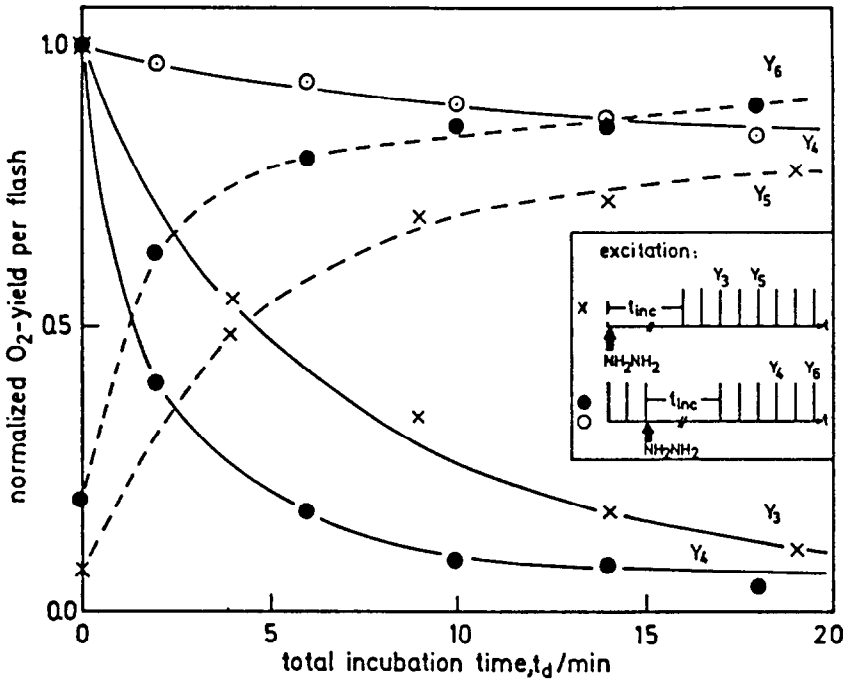

Fig. 3. Normalized oxygen yield of n-th flash, Y_n, as a function of the total incubation time between addition of 1 mM NH_2NH_2 and the actinic flash train in thylakoids at pH 7.2. For experimental details see section 2. The data were normalized on the extent of Y_3 and Y_4 in the oscillation pattern of control thylakoids without NH_2NH_2 addition at $t_d = 0$ (the open symbols indicate the decay of Y_4 in control samples illuminated with three pre-flashes, Y_3 remains constant in samples illuminated with one pre-flash).

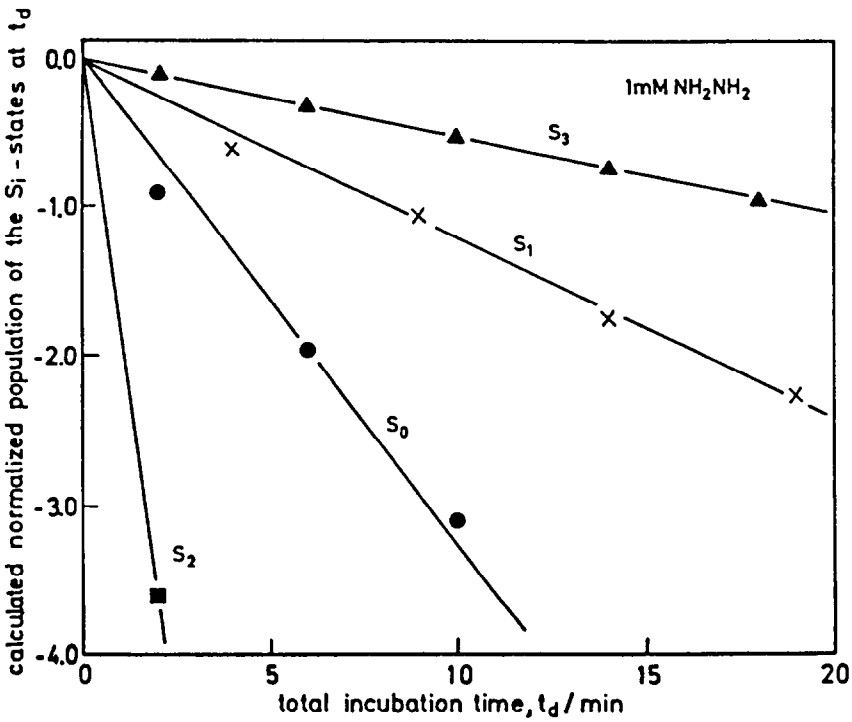

Fig. 4. Semi-logarithmic plot (ln scale) of the calculated depopulation of the redox states S_i induced by 1 mM NH_2NH_2. For experimental details see section 2. The data shown represent the difference of the ln values of the corresponding S_i state populations in the presence and absence of NH_2NH_2, respectively. For further details see text.

previous report on the NH_2OH-induced S_2 and S_3 decay [17].

It was recently shown that in samples with zero population of S_2 and S_3, the compound NH_2NH_2 introduces a two-electron pool capacity regardless of the population of S_0 and S_1 [24], giving rise to the formal hole-storage states 'S_{-1}' and 'S_{-2}' (a more complex pattern arises for NH_2OH [17]). It is, therefore, mechanistically interesting to compare the NH_2NH_2-induced S_2 and S_3 reduction kinetics with the transformation rates of S_0 (into 'S_{-2}') and S_1 (into 'S_{-1}') in dark-adapted thylakoids [24]. If the samples are kept in state S_1, then the maximum of the oxygen-yield pattern is shifted from the 3rd to the 5th flash. Accordingly, the decline of the oxygen yield due to the 3rd flash, Y_3, and the concomitant rise of Y_5 reflects the reduction of the proposed two-electron pool by NH_2NH_2. Likewise, the decline of the oxygen yield due to the 4th flash, Y_4, and the corresponding increase of Y_6 in samples pre-illuminated with 3 flashes indicate the transformation of S_0 into a formal state, 'S_{-2}'. The data obtained are shown in Fig. 3. As expected, the decay of Y_3 kinetically corresponds with the rise of Y_5. Likewise, an analogous dependence is observed for Y_4 and Y_6.

The overall kinetics in Figs 2 and 3 are due to different competing decay processes, and therefore an appropriate separation procedure is required to extract the specific NH_2NH_2 effects. The rate constants of the NH_2NH_2-induced S_2 and S_3 decline can be calculated, if this reaction introduces an additional (exogenous) decay without an effect on the reaction with the endogenous reductants. In samples preilluminated with one flash, the contribution of the small extent of the fast endogenous decay (with Y_D as donor) can be neglected. Accordingly, one obtains:

$$k_i(NH_2NH_2) = k_i^{\Sigma} - k_i^{control} \quad (1)$$

where i = 2, 3 and k_i^{Σ}, $k_i^{control}$ = rate constants of the measured $S_2(S_3)$ decay in the presence and absence of NH_2NH_2, respectively. As the state S_1 is stable in the control, k_1^{Σ} directly reflects the rate constant of the transformation into the formal redox state 'S_{-1}', while in samples populated in S_0 by 3 preflashes, a small correction is required due to slow S_0 reoxidation in the control. The kinetics of the transformation of S_0, S_1, S_2 and S_3 by 1 mM NH_2NH_2 are shown in Fig. 4 as a semilogarithmic plot. In the case of S_2, the reaction is too fast to be resolvable but the other kinetics appear to be monoexponential. Three interesting features emerge from Fig. 4: (a) the rate of the NH_2NH_2-induced decay of S_2 is at least 25 times faster than of S_3; (b) the transformation rate of S_0 into 'S_{-2}' by NH_2NH_2 exceeds that of S_1 → 'S_{-1}' by a factor of about 2.5–3, and (c) the reaction of S_3 with NH_2NH_2 is the slowest process. Analogous differences between S_2 and S_3 were observed for NH_2OH (data not shown) in qualitative correspondence with latest findings in etiolated oat plastids [25].

4. DISCUSSION

This study revealed that in intact thylakoids the reactivity of the PS II donor side towards NH_2NH_2 exhibits the following order of dependence on the redox state S_i of the water-oxidizing enzyme: $S_3 < S_1 < S_0 << S_2$. Of special mechanistic relevance is the striking kinetic difference of the susceptibility towards NH_2NH_2 between S_2 and S_3 as opposed by the very similar decay rates of these states due to electron transfer from endogenous electron donors (Y_D, PS II acceptor side). Regardless of the mode of NH_2NH_2-interaction (for a recent discussion see ref. [26]), the redox state S_3 is shown to be significantly protected from dissipation by exogenous reductants despite its high overall oxidation level. This implies that S_3 is characterized by a very special electronic configuration and/or nuclear geometry. In the simplest case, the redox transitions S_2 → S_3 would lead to a conformational change that establishes a barrier against exogenous reductants like NH_2NH_2 or NH_2OH. In addition to that, the formation of S_3 could also comprise an electronic redistribution of the oxidizing equivalents between manganese and ligands, including the water as substrate. The latter process might lead to a peroxidic state [27] which is much less susceptible to exogenous reductants than manganese in a high valence state as in S_2. The contribution of electronic and structural effects to the special properties of S_3 remain to be clarified. Related to this problem is the question about the functional relevance of the 'shielding' of S_3 from substances like NH_2NH_2 (NH_2OH), especially in respect to the accessibility to water (for a discussion of this problem see ref. [28]). It is interesting to note that also S_1 is kinetically less susceptible to NH_2NH_2 than S_0. This might indicate structural differences between S_0 and S_1.

In general, the present report shows that kinetic studies on the interaction with exogenous substances can provide interesting information on the properties of the redox states S_i of the water-oxidizing enzyme system.

Acknowledgements: The authors would like to thank E. Haag and Dr T. Wydrzynski for critical reading of the manuscript. The financial support by the Deutsche Forschungsgemeinschaft (Re 354/11-1) is gratefully acknowledged.

REFERENCES

[1] Babcock, G.T. (1987) in: New Comprehensive Biochemistry (Amesz, J. ed.) pp. 125–158, Elsevier, Amsterdam.
[2] Renger, G. (1987) Angew. Chem. Int. Ed. 26, 643–660.
[3] Rutherford, A.W. (1989) Trends Biochem. Sci. 14, 227–232.
[4] Hansson, Ö. and Wydrzynski, T. (1990) Photosynth. Res. 23, 131–161.
[5] Debus, R., Barry, B.A., Sithole, I., Babcock, G.T. and McIntosh, L. (1988) Biochemistry 27, 9071–9074.

[6] Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457-475.
[7] Vermaas, W.F.J., Renger, G. and Dohnt, G. (1984) Biochim. Biophys. Acta 704, 194-202.
[8] Debus, R.J., Barry, B.A., Babcock, G.T. and McIntosh, L. (1988) Proc. Natl. Acad. Sci. USA 85, 8427-8430.
[9] Vermaas, W.F.J., Rutherford, A.W. and Hansson, Ö. (1988) Proc. Natl. Acad. Sci. USA 85, 8477-8481.
[10] Robinson, H.H. and Crofts, A.R. (1983) FEBS Lett. 153, 221-226.
[11] Rutherford, A.W., Renger, G., Koike, K. and Inoue, Y. (1984) Biochim. Biophys. Acta 767, 548-556.
[12] Messinger, J. and Renger, G. (1990) in: Current Research in Photosynthesis (Baltscheffsky, M. ed.) Vol. 1, pp. 849-852, Kluwer, Dordrecht.
[13] Vass, I., Deak, Z. and Hideg, E. (1990) Biochim. Biophys. Acta 1017, 63-69.
[14] Styring, S. and Rutherford, A.W. (1987) Biochemistry 26, 2401-2405.
[15] Renger, G., Bouges-Bocquet, B. and Delosme, R. (1973) Biochim. Biophys. Acta 292, 796-807.
[16] Andreasson, L.E. and Hansson, Ö. (1987) in: Progr. in Photosynth. Res. (Biggins, J. ed.) Vol. 1, pp. 503-510, Nijhoff, Dordrecht.
[17] Hanssum, B. and Renger, G. (1985) Biochim. Biophys. Acta 810, 225-234.
[18] Bouges, B. (1971) Biochim. Biophys. Acta 234, 102-112.
[19] Koike, H., Hanssum, B., Inoue, Y. and Renger, G. (1987) Biochim. Biophys. Acta 893, 524-533.
[20] Winget, G.H., Izawa, S. and Good, N.E. (1965) Biochem. Biophys. Res. Commun. 21, 438-441.
[21] Vass, I., Deak, Z., Jegerschold, C. and Styring, S. (1990) Biochim. Biophys. Acta 1018, 41-46.
[22] Joliot, P. (1972) Methods Enzymol. 24, 123-134.
[23] Joliot, P. and Kok, B. (1975) in: Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 387-412, Academic Press, New York.
[24] Renger, G., Messinger, J. and Hanssum, B. (1990) in: Current Research in Photosynthesis (Baltscheffsky, M. ed.) Vol. 1, pp. 845-848, Kluwer, Dordrecht.
[25] Franck, F. and Schmid, G.H. (1989) Biochim. Biophys. Acta 977, 215-218.
[26] Renger, G., Bader, K.P. and Schmid, G.H. (1990) Biochim. Biophys. Acta 1015, 288-294.
[27] Renger, G. (1978) in: Photosynthetic Oxygen Evolution (Metzner, H. ed.) pp. 229-248, Academic Press, London.
[28] Renger, G. and Wydrzynski, T. (1991) Biol. Metals (in press).