Structure–Function Relations in Photosystem II. Effects of Temperature and Chaotropic Agents on the Period Four Oscillation of Flash-Induced Oxygen Evolution†

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ABSTRACT: The characteristic period four oscillation patterns of oxygen evolution induced by a train of single-turnover flashes were measured in dark-adapted samples as a function of temperature and upon addition of chaotropic agents. The following results were obtained: (a) Within the range of $0 < \vartheta < 35$ °C, the ratio of the oxygen yield induced by the 4th and 3rd flashes of the train, Y_4/Y_3, and the oxygen yield induced by the 2nd flash, Y_2, exhibit similar dependencies on the temperature in isolated thylakoids, PS II membrane fragments, and inside-out vesicles. (b) Below a characteristic temperature ϑ_c of 20–25 °C, the values of Y_4/Y_3 and Y_2, which reflect (at constant S_0 dark population) the probabilities of misses and double hits, respectively, remain virtually independent of temperature, whereas above ϑ_c these parameters increase. (c) The dark decays of S_2 and S_3 via fast and slow kinetics due to reduction of the water oxidase by Y_D and other endogenous electron donor(s), respectively, exhibit comparatively strong temperature dependencies in thylakoids with the following activation energies: $E_A(S_2^{fast}) = 55$ kJ/mol, $E_A(S_3^{fast}) = 50$ kJ/mol, $E_A(S_2^{slow}) = 85$ kJ/mol, and $E_A(S_3^{slow}) = 75$ kJ/mol. The activation energy of S_0 oxidation to S_1 by Y_D^{ox} was found to be markedly smaller with a value of $E_A(S_0) = 30$ kJ/mol. (d) Incubation with chaotropic agents at concentrations which do not significantly impair the oxygen evolution capacity leads to modifications of the oscillation pattern with remarkable differences for various types of agents: Tris and urea are practically without effect; guanidine hydrochloride affects Y_4/Y_3 in a similar way as elevated temperature but without significant changes of Y_2 and the decay kinetics of S_2 and S_3; and anions of the Hofmeister series (SCN^-, ClO_4^-, I^-) cause a drastic destabilization of Y_D^{ox}. Possible structure–function relations of the PS II complex are discussed on the basis of these findings.

Photosynthetic water oxidation to dioxygen and the coupled release of four protons into the thylakoid lumen take place within a manganese-containing functional unit referred to as the water oxidase. This unit, as a part of the membrane integral PS II[1] complex, is regulated by extrinsic proteins and other cofactors such as Ca^{2+} and Cl^-. 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 Kok cycle (Kok et al., 1970), is energetically driven by $P680^+$ as the oxidant, which is formed during the primary step of light-induced charge separation within the PS II reaction center [for a recent review, see Renger (1992)]. A redox component, Y_Z, acting as an intermediate electron carrier functionally connects the water oxidase with $P680^+$. Component Y_Z has been identified by site-directed mutagenesis in *Synechocystis* sp. PCC 6803 as a tyrosine (Tyr-161) of polypeptide D1 (Debus et al., 1988a; Metz et al., 1989). Polypeptides D1 and D2 form a heterodimer which provides the apoprotein of the PS II reaction center (Michel & Deisenhofer, 1988). The kinetics of the elementary reactions in the overall reaction sequence of PS II have been resolved in great detail by various spectroscopic techniques [for recent reviews, see Babcock (1987), Renger (1987a), and Rutherford et al. (1992)].

The four-step univalent oxidative pathway within the water oxidase can be summarized by the formula

$$Y_Z^{ox}(S_i) + m_i H_2O \rightarrow Y_Z(S_{i+1}) + n_i H^+ + \delta_{i3} O_2 \quad (1)$$

where S_i symbolizes the redox state of the water oxidase, with $i = 0, ..., 3$ representing the number of accumulated oxidizing redox equivalents, n_i describes the noninteger stoichiometry [see Rappaport and Lavergne (1991)] of net H^+ release, m_i is the net uptake of m water molecules coupled with one-electron abstraction from the water oxidase in redox state S_i (with $\sum_{i=0}^{3} m_i = 2$), and δ_{ij} is the Kronecker symbol ($\delta_{i3} = 1$ for $i = 3$, otherwise zero). Equation 1 also tacitly implies that redox state S_4 rapidly transfers under dioxygen formation to S_0 and therefore cannot be detected as a kinetically separable component, i.e., $i + 1 = 4$ has to be read in eq 1 as zero.

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[1] Abbreviations: ADRY, acceleration of the deactivation reaction of water splitting system; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; D1 and D2, polypeptides of the phototsystem II core; FWHM, full width at half-maximum; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PS II, photosystem II; P680, primary electron donor in PS II; Q_A and Q_B, primary and secondary plastoquinone acceptors of PS II; R_D and R_Z, distance of the water oxidase to tyrosines Y_D and Y_Z, respectively; S_i, redox state i of the water oxidase; Tricine, *N*-tris(hydroxymethyl)methylglycine; ϑ_c, critical temperature; Y_Z, redox-active tyrosine of polypeptide D1 that acts as an electron donor to $P680^+$; Y_D, redox-active tyrosine of polypeptide D2; Y_i, normalized oxygen yield due to the ith flash of a flash train.

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In addition to Y_Z as the unique oxidant, other endogenous redox groups can interfere with different states S_i (i = 0, 2, 3) of the water oxidase. In analogy to Y_Z in D1, the D2 polypeptide also contains a redox-active tyrosine residue referred to as Y_D [identified as Tyr-160 in *Synechocystis* sp. PCC 6803 (Debus et al., 1988b; Vermaas et al., 1988) but Tyr-161 in spinach (Debus, 1992)]. Although, according to structural modeling, Y_Z and Y_D are almost symmetrically arranged with respect to P680 (Svensson et al., 1990; Ruffle et al., 1992), Y_D is not involved in the main electron pathway. The markedly lower redox potential of the couple Y_D/Y_D^{ox} (Boussac & Etienne, 1984) does not permit S_i state oxidation by Y_D^{ox} for $i \geq 1$, but rather leads to a reduction of S_2 and S_3 by Y_D. Only S_0 can be oxidized to S_1 via a rather slow reaction (Styring & Rutherford, 1987).

In addition, the redox states S_2 and S_3 also undergo a slow decay into S_1 via back-reactions with the acceptor side, probably involving Y_Z and P680 as intermediates as deducted from delayed light emission (Rutherford & Inoue, 1983) and thermoluminescence measurements (Rutherford et al., 1984). Interaction with other endogenous reductants may be possible as well. In view of this complexity, the slow reduction of S_2 and S_3 by all endogenous electron donors other than Y_D will be described as interaction with unspecified reductant(s).

Apart from the action of these endogenous groups, the reaction pattern can also be selectively modified by the addition of specifically interacting exogenous compounds like hydrophobic ADRY-type substances (Renger, 1972; Hanssum & Renger, 1985) or hydrophilic redox-active amines like NH_2-OH and NH_2NH_2 [see Messinger et al. (1991) and references cited therein].

Despite the detailed kinetic information on the reaction pattern, key mechanistic questions still remain to be answered [for a list, see Renger (1987b)]. Likewise, the structure of the water oxidase and the nature of its protein matrix are not yet resolved [for a detailed review, see Debus (1992)]. Among these problems, the entry of water into the redox cycle (symbolized by unknown m_i in eq 1) and the mode of oxygen–oxygen bond formation (probably at the level of a peroxidic configuration) are of special relevance (Renger & Wydrzynski, 1991). Another key point is the role of the apoprotein. The unique properties of proteins not only permit an ideal fine tuning of the reaction coordinates of the sequential redox steps summarized in eq 1 but also ensure a well-controlled and protected performance of water cleavage, which takes place at high oxidation potentials within an environment highly susceptible to triggering of oxidative degradation. Recent studies analyzed the reaction coordinates of $P680^+$ reduction by Y_Z and the univalent redox steps in the water oxidase by measuring the temperature dependence of the rate constants (Koike et al., 1987; Renger et al., 1989a; Renger & Hanssum, 1992). In this article, temperature effects on the interaction of the water oxidase with Y_D and the unspecified endogenous reductant(s) were investigated. Furthermore, changes of the flash-induced oxygen evolution pattern were analyzed which are induced by structural modifications due to the interaction with a class of solutes referred to as chaotropic agents.

MATERIALS AND METHODS

Standard procedures were used to isolate thylakoids from market spinach (Winget et al., 1965), PS II membrane fragments [Berthold et al. (1981), with slight modifications described in Völker et al. (1985)], and inside-out vesicles (Akerlund & Anderson, 1983). After the final isolation step, thylakoids and PS II membrane fragments were resuspended in a weakly buffered medium (400 mM sucrose, 20 mM NaCl, 5 mM $MgCl_2$, and either 10 mM Mes/NaOH (pH 6.5) or 5 mM Hepes/NaOH (pH 7.0)) to chlorophyll concentrations of about 4 mg/mL. Samples were frozen in small aliquots in liquid nitrogen and stored at −80 °C until use. Inside-out vesicles were resuspended in the same medium but with 50 mM Mes/NaOH (pH 6.5) and at chlorophyll concentrations of about 1 mg/mL. Before the measurements the samples were thawed and $Y_D^{ox}(S_1)$ samples were obtained by excitation with one flash and subsequent dark incubation on ice, as described previously (Messinger & Renger, 1990).

Bio-Beads treatment of PS II membrane fragments was performed essentially according to the procedure described by Holloway (1973). A suspension of 100 μL (PS II membrane fragments at 1 mg Chl/mL, 300 mM mannitol, 20 mM NaCl, 10 mM $MgCl_2$, and 50 mM Hepes/NaOH (pH 7.2)) was incubated with 5 mg of washed SM-2 Bio-Beads (Bio-Rad) and kept on ice for about 1 h in the dark under gentle shaking.

Flash-induced O_2 oscillation patterns were measured with a modified Joliot-type electrode (Joliot, 1972) that keeps the temperature at the electrode and the buffer reservoir constant within ±0.2 °C. The pH of the flow buffer (see the figure legends) was adjusted at each temperature to the indicated values in order to eliminate temperature-dependent pH shifts. The samples were diluted to a chlorophyll concentration of 1 mg/mL with an aliquot of the flow buffer (cooled on ice), and 10 μL of this suspension was transferred to the Joliot-type electrode. All sample handlings were performed in dim green light. The samples were incubated for 2 min on the Pt electrode before the measurements were started. This time was found to be sufficiently long enough for complete thermal equilibration of the 10-μL samples and simultaneously short enough to prevent serious denaturation effects at elevated temperatures. The polarization current of −600 or −750 mV was switched on 30 s before excitation with a train of short (FWHM = 3 μs), saturating Xe flashes (General Radio Stroboslave 1539A) separated by a dark time of 500 ms.

The probabilities of misses, double hits, and the apparent S_0 populations were determined by a least-squares fit method comparing the relative oxygen yields of the first 12 flashes of the train with calculated sequences (on the basis of the conventional Kok model; Kok et al., 1970) as outlined in Messinger et al. (1991). The S_2 and S_3 lifetimes were measured in the conventional way (Joliot & Kok, 1975) by exciting dark-adapted samples with one (S_2 formation) or two (S_3 formation) preflashes and monitoring the O_2 yield pattern induced by a flash train (2 Hz) given at various dark times t_d after the preflashes. These patterns were deconvoluted into normalized S_i state populations within the framework of the conventional Kok model by the use of a least-squares fit method, taking the misses and double hits of the normal sequence (2 Hz) as fixed values. The rate constants were derived from a semilogarithmic plot of the normalized S_2 or S_3 populations as a function of t_d.

The S_0 lifetimes were measured in samples with enriched S_0 populations by giving three preflashes (at 5 Hz) to Y_D^{ox} (S_1) thylakoids and varying the dark time t_d between the last preflash and the subsequent train of 15 flashes at 2 Hz. Effects of fast and slow S_2 and S_3 decay and the slow S_0 oxidation by Y_D^{ox} were taken into account by using a "kinetic" Kok model to calculate the changes of S_i state population during the dark times between the flashes (of the flash trains and during t_d). The rate constants for S_2 and S_3 decay were directly determined experimentally (vide supra), while the rate constant for the S_0 oxidation by Y_D^{ox} was the fit parameter for the

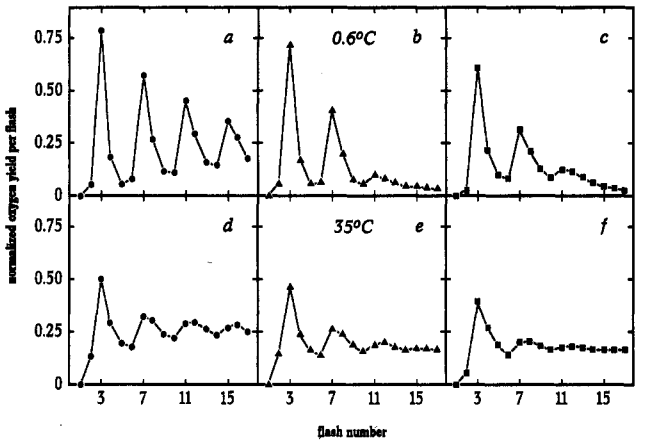

FIGURE 1: Normalized oxygen yield as a function of flash number in dark-adapted spinach ($Y_D^{ox}(S_1)$) thylakoids (●), inside-out vesicles (▲), and PS II membrane fragments (■) illuminated by a train of single-turnover flashes at 0.6 °C/pH 6.5 (a–c) and 35 °C/pH 6.5 (d–f). The flow buffer contained 20 mM NaCl, 5 mM $MgCl_2$, and 50 mM MES (pH 6.5). The oxygen yield of each flash was normalized to one-third of the sum of the first 12 flashes (thylakoids) or to the sum of flashes 3–6 (inside-out vesicles and PS II membrane fragments) of the oscillation pattern. The latter normalization was necessary due to the limited pool capacity of the latter two sample types. Application of this normalization to thylakoids did not affect the results of a and d (data not shown).

description of the oscillation patterns measured at different dark times t_d (the details of this fit procedure will be described in a forthcoming paper).

RESULTS

Effects of Temperature on the Oscillation Pattern of Flash-Induced Oxygen Evolution. Figure 1 depicts the characteristic period four oscillation of the oxygen yield induced by a flash train in dark-adapted samples of isolated thylakoids, detergent-free inside-out vesicles, and Triton X-100 PS II membrane fragments at 0.6 °C (top traces) and 35 °C (bottom traces), respectively. At first glance, three striking phenomena are observed: (a) at 35 °C the oscillations are much more damped in all three sample types; (b) the normalized oxygen yield due to the second flash, symbolized by Y_2, is markedly higher at 35 °C; and (c) in contrast to isolated thylakoids, the O_2 yields fade out after two periods in PS II membrane fragments and inside-out vesicles. This difference is markedly less pronounced at 35 °C.

Within the framework of Kok's model, and taking into account the observation that practically all oxygen-evolving complexes attain the redox state S_1 after sufficiently long dark adaptation (Vermaas et al., 1984), the first two effects can be phenomenologically explained by an increase of the probabilities of misses (α) and double hits (β) at higher temperatures. Phenomenon c can be explained by the limited electron acceptor pool size in PS II membrane fragments and inside-out vesicles.

In order to analyze these temperature effects in more detail, the oscillation patterns were measured at intervals of about 3 °C within the range of 0–40 °C. For a characterization of the thermally induced phenomena, the following quantities were used as qualitative measures of the parameters, α and β, and the effective pool size: (i) the ratio of the oxygen yields of the 4th and 3rd flashes, respectively, Y_4/Y_3, as a measure of the probability of misses α (vide infra); (ii) the value Y_2 as a measure of double hits β; and (iii) the ratio $\sum_{i=11}^{14} Y_i / \sum_{i=3}^{6} Y_i$ reflecting the effective pool size (Y_i = normalized oxygen yield due to the ith flash). The data gathered from

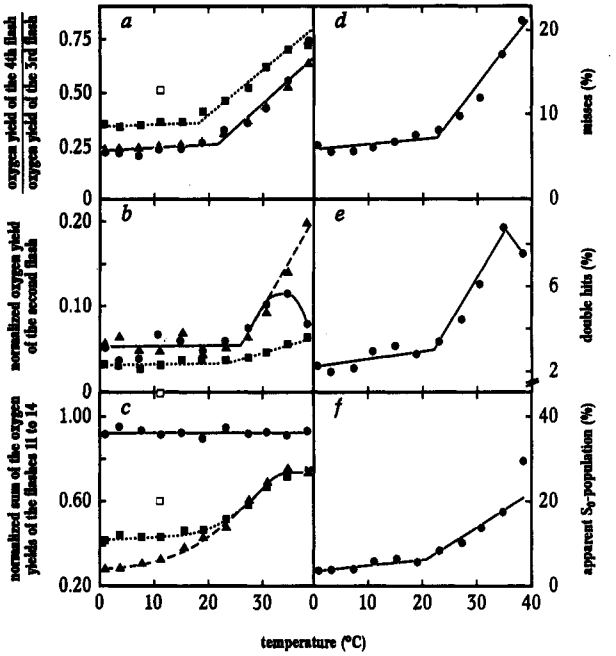

FIGURE 2: Characteristic parameters calculated from the period four oxygen yield pattern induced by a train of single-turnover flashes in dark-adapted spinach thylakoids (●), inside-out vesicles (▲), and PS II membrane fragments (■) as a function of temperature. The data were obtained from measurements of oscillation patterns analogous to those shown in Figure 1. Left side: (a) ratio of the oxygen yields of the fourth and the third flashes, Y_4/Y_3; (b) normalized sum of the oxygen yields of the second flash; (c) normalized sum of the oxygen yields of the 11th–14th flashes reflecting the relative acceptor pool capacity. For each oscillation pattern the values were normalized to the sum of the oxygen yields of flashes 3–6. The open squares represent the results obtained in PS II membrane fragments that were heated for 3 min outside the electrode at 40 °C and then incubated on ice for 1 h before the measurement. Right side: (d) probability of misses; (e) double hits; and (f) the apparent S_0 population of spinach thylakoids as a function of temperature. The same oscillation patterns as in a–c were used to calculate α, β, and $[S_0]$ with the conventional static Kok model.

the experimentally measured oscillation patterns are shown in Figure 2 (left side). The temperature dependence of Y_4/Y_3 and Y_2 exhibits, for all three types of samples, practically the same pattern with a characteristic transition temperature, ϑ_c, of 20–25 °C. At values below ϑ_c, the quantities Y_4/Y_3 (figure 2a) and Y_2 (figure 2b) are virtually independent of temperature, while above ϑ_c a remarkable increase is observed with increasing temperature.

A more detailed inspection reveals marked differences between Triton X-100 PS II membrane fragments and the other two types of detergent-free preparations, i.e., isolated thylakoids and inside-out vesicles. In PS II membrane fragments, the values of Y_4/Y_3 are higher and those of Y_2 are lower than those in the other two preparations for $0 < \vartheta < 35$ °C. The differences in these parameters can be, at least partly, removed by incubation of PS II membrane fragments with Bio-Beads, as described in Materials and Methods (data not shown). Therefore, the effects on Y_4/Y_3 and Y_2 could be ascribed to the presence of detergent which leads to acceptor side modifications due to the Triton X-100 treatment in the isolation procedure of PS II membrane fragments (see Discussion).

The effective acceptor pool size, as given by $\sum_{i=11}^{14} Y_i / \sum_{i=3}^{6} Y_i$, exhibits a different temperature dependence. In isolated thylakoids this quantity is constant over the whole range from 0 to 40 °C, whereas in PS II membrane fragments and inside-out vesicles the limited pool capacity increases at

higher temperatures. At moderate temperatures (10–35 °C), the increase is assumed to be mainly due to the faster reoxidation of PQH_2 and the increasing miss parameter α, while at higher temperatures the further increase is dominated by the decrease of the number of functionally competent water-oxidizing complexes (vide infra).

As the quantities Y_4/Y_3 and Y_2 can provide only a qualitative measure of α and β (if the ratio of S_0/S_1 state dark population remains independent of temperature and the fast reduction of S_2 and S_3 by Y_D can be neglected), the values of α and β were determined quantitatively within the framework of the conventional Kok model (Kok et al., 1970). This calculation was performed only for the data of isolated thylakoids because the limited effective acceptor pool capacity of PS II membrane fragments and inside-out vesicles prevents a sufficiently reliable determination of α and β. The results obtained are shown on the right side of Figure 2 (traces d and e). A comparison with the data in Figure 2a,b readily shows that the temperature dependence of Y_4/Y_3 and Y_2 reflects the thermal effects on α and β, respectively. The analysis also reveals that changes of the apparent dark population ratio S_0/S_1 have to be taken into account. As expected, in $Y_D^{ox}(S_1)$ samples the apparent S_0 value is small at lower temperatures, as is shown in Figure 2f. At higher temperatures, however, the apparent S_0 population exceeds the values that are expected on the basis of the normalized extent and the rate constants of the fast S_2 and S_3 decay in these samples (vide infra).

In general, the temperature range where the above-mentioned effects can be analyzed is limited due to the thermal degradation and denaturation of the samples. Previous measurements of the absolute average oxygen yield per flash with a Clark-type electrode have shown that dark incubation (3 min) of isolated thylakoids at elevated temperatures causes an irreversible loss of the oxygen evolution capacity (Renger et al., 1989b). The onset of this effect is in the range of 35–40 °C. Thermal inactivation of oxygen evolution (Kimimura & Katoh, 1972) was shown to be accompanied by loss of manganese (Wydrzynski & Sauer, 1980) and protein release (Nash et al., 1985) and is therefore ascribed to a destruction of the water-oxidizing complex. On the basis of a shift of 5–10 °C to higher temperatures in D_2O suspensions, 6–7 hydrogen bonds were inferred to be of structural relevance for a functionally competent water oxidase (Renger et al., 1989b).

If all or no blockage of this system would be the only thermal effect on PS II, the values of Y_4/Y_3 and Y_2 should remain unaffected, while the effective pool size in inside-out vesicles and PS II membrane fragments would be expected to increase, according to recent results obtained in the presence of DCMU (Renger et al., 1989c). In order to address this point, experiments were performed with samples preincubated in the dark for 3 min at a certain temperature followed by 1 h of dark incubation on ice and subsequent transfer to the Joliot-type electrode. The oscillation patterns of these samples were measured at 10 °C. The results obtained reveal that incubation temperatures up to about 30 °C affect neither Y_4/Y_3 and Y_2 nor the effective acceptor pool capacity (data not shown). However, at higher temperatures irreversible effects arise. This is illustrated in Figure 2a–c for the case of PS II membrane fragments which were dark-incubated for 3 min at 40 °C. The results symbolized by open squares clearly show that the probability of misses markedly increases while that of double hits drops down to zero. As expected, the effective acceptor pool size also increases due to this treatment. These findings provide unambiguous evidence for irreversible structural changes in PS II induced by dark incubation at elevated temperatures. Accordingly, for the following considerations only the results below about 35 °C will be taken into account because they reflect reversible thermal effects under our experimental conditions.

Table I: Normalized Extents (a_f, a_s)[a] and Half-Life Times ($t_{1/2}^f$ and $t_{1/2}^s$) of the Fast (f) and Slow (s) Phases of S_2 and S_3 Decays at Different Temperatures in Isolated Thylakoids at pH 7.0

	S_2 decay				S_3 decay			
temperature (°C)	a_f	$t_{1/2}^f$ (s)	a_s	$t_{1/2}^s$ (s)	a_f	$t_{1/2}^f$ (s)	a_s	$t_{1/2}^s$ (s)
5	0.26	46	0.74	420	0.21	21	0.79	460
10	0.31	25	0.69	270	0.27	17	0.73	270
15	0.36	15	0.64	160	0.24	9	0.76	160
20	0.35	11	0.65	75	0.28	7	0.72	94
25	0.38	8	0.62	40	0.30	4.5	0.70	56
30	0.37	5	0.63	27	0.30	4	0.70	40
35	0.38	4	0.62	14	0.22	2.5	0.78	20

[a] The magnitudes of a_f and a_s depend on the storage time of the thylakoids at −80 °C (Messinger et al., 1991).

Activation Energies of S_2 and S_3 Decay and S_0 Oxidation by Endogenous Redox Components. An inherent property of the water oxidase is the relaxation of the redox states S_2 and S_3 to S_1 by interaction with the redox-active tyrosine Y_D of polypeptide D2 and the PS II acceptor side, as well as the oxidation of redox state S_0 by Y_D^{ox}. It therefore appears to be worth analyzing the temperature dependence of these reactions. The decay kinetics of S_2 and S_3 can be determined by varying the time between the first and second as well as between the second and third flashes, respectively, of the sequence as outlined previously [for a review, see Joliot and Kok (1975)]. In general, a biphasic decay is observed where the fast phase reflects the reduction of S_2 and S_3 by Y_D and the slow phase represents the kinetics of the S_2 and S_3 decay by electron donation from the acceptor side and other endogenous reductants. The data obtained from measurements in the range between 5 and 35 °C are summarized in Table I.

A remarkable increase of the S_2 and S_3 stability is observed with decreasing temperatures for both the fast and the slow decays. This finding shows that at low temperatures the oscillation of the oxygen yield persists even for rather long dark times (up to a few minutes!) between the flashes, as is illustrated in Figure 3. Accordingly, measurements at lower temperatures are much more suitable for analysis of the period four oscillation of the redox states, S_i.

The rate constant of the S_0 oxidation by Y_D^{ox} was determined by varying the time between the third and fourth flashes of the sequence. The determination of the rate constants of the S_0 oxidation by Y_D^{ox} requires that the data fit with a Kok model that includes changes of the S_i states during the dark time between the flashes, because the analysis within the framework of a "static" conventional Kok model is difficult for the following reasons: (i) the increase of Y_6 (the number 6 includes the three preflashes), reflecting the increase of the S_1 population due to S_0 oxidation by Y_D^{ox}, is affected by the decay of the partial S_2 and S_3 populations that are concomitantly generated by the three preflashes, and (ii) the decrease of the 7th flash, as a measure of declining S_0 population caused by the reaction $Y_D^{ox}(S_0) \rightarrow Y_D(S_1)$, becomes significantly retarded due to the interaction of Y_D with the S_2 and S_3 states during the flash sequence. This phenomenon is especially pronounced at elevated temperatures. Both effects were taken into account by fitting the data with a kinetic Kok model, as briefly described in Materials and Methods (a detailed description will be presented elsewhere; U. Wacker, J. Messinger, and G., Renger, in preparation). This procedure

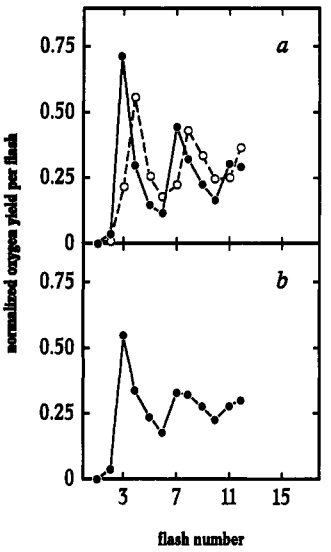

FIGURE 3: Normalized oxygen yield as a function of flash number in dark-adapted spinach thylakoids illuminated by a train of single-turnover flashes at dark times between the flashes of 60 (a) and 180 s (b). The measurements were performed at 0.1 °C and pH 7.2 (300 mM mannitol, 20 mM $CaCl_2$, 10 mM $MgCl_2$, and 50 mM Hepes). In order to avoid the influence of sedimentation effects on the pattern, the samples were incubated for 10 min on the electrode before the measurements. The polarization current of −750 mV was switched on 30 s before each flash: (●) $Y_D^{ox}(S_1)$ thylakoids; (○) thylakoids without preflash treatment.

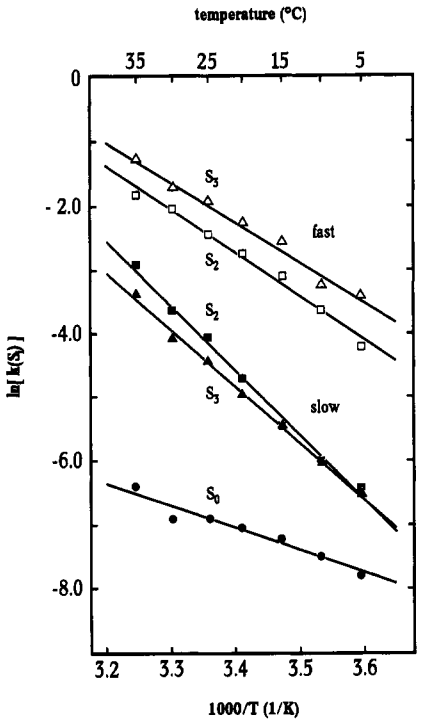

FIGURE 4: Semilogarithmic plot of the rate constants of the slow (■, ▲) and the fast (□, △) S_3 (triangles) and S_2 (squares) decays and of the dark oxidation of S_0 by Y_D^{ox} (●) as a function of reciprocal temperature in spinach thylakoids at pH 7.0 (300 mM sucrose, 20 mM NaCl, 5 mM $MgCl_2$, and 50 mM Hepes).

permits a reliable determination of the rate constants for the S_0 oxidation by Y_D^{ox}. The half-life times obtained for this reaction vary between 7 (35 °C) and 30 min (5 °C).

Figure 4 shows an Arrhenius-type semilogarithmic plot of the rate constants determined for the above-mentioned reactions as a function of reciprocal temperature. The activation energies calculated from this plot are summarized in Table II. Recently, very similar values were reported for the activation energies of S_2 and S_3 decay in the temperature range of 8–37 °C (Vass et al., 1990). The data in Figure 4 do not exhibit any clear breakpoint in the activation energy at a characteristic transition temperature. This finding suggests that the rate-limiting step of these reactions is not affected by thermally induced structural changes in PS II.

Table II: Activation Energies of Dark Relaxation Reactions in the Water Oxidase

reaction type	activation energy
S_2 and S_3 reaction by Y_D	$E_A(S_2^{fast}) = 55$ kJ/mol
	$E_A(S_3^{fast}) = 50$ kJ/mol
S_2 and S_3 reduction by	$E_A(S_2^{slow}) = 85$ kJ/mol
other endogenous electron donors	$E_A(S_3^{slow}) = 75$ kJ/mol
S_0 oxidation by Y_D^{ox}	$E_A(S_0) = 30$ kJ/mol

On the other hand, the characteristic changes at ϑ_c of different parameters summarized in Figure 2 could be indicative of thermally induced reversible structural changes (irreversible effects will not be considered; vide supra) of the PS II protein complex. In order to check this idea, it appears worthwhile to analyze the effects of structure-modifying substances like chaotropic agents.

Effects of Chaotropic Agents. Chaotropic agents are chemicals which are known to destabilize native protein structures by affecting the protein–water interactions [for a review, see Collins and Washabaugh (1985)]. They can also alter the properties of lipid membranes (Hatefi & Hanstein, 1969; Sanderson et al., 1991). In an attempt to mimic the thermally induced effects by chaotropic agent induced structural changes, the oscillation patterns of flash-induced oxygen evolution were measured at 20 °C and different concentrations of these substances. It has to be emphasized that chaotropic agents at higher concentrations destroy the oxygen evolution capacity (Lozier et al., 1971), often under release of the extrinsic regulatory 33-kDa polypeptide (Akerlund & Jansson, 1981; Murata et al., 1983). Therefore, the experiments of this study were performed at concentrations of the chaotropic agents which are sufficiently low to avoid significant detrimental effects.

Figure 5 shows typical oscillation patterns measured at 20 °C in a control (a) and samples incubated for 1 h in the dark on ice with either 100 mM NaSCN (b), 100 mM guanidine hydrochloride (c), or 400 mM urea (d). An inspection of the data readily reveals marked differences among the chaotropic agents (NaSCN, guanidine hydrochloride, urea) in terms of their mode of interaction with the PS II complex at concentrations which do not significantly affect the oxygen evolution capacity. A comparison of Figure 5c and Figure 1d reveals that guanidine hydrochloride appeared to be most promising for mimicking thermally induced reversible structural changes which affect the probabilities of misses.

In Figure 6, the oscillation patterns obtained in isolated spinach thylakoids at three selected temperatures above ϑ_c (left side) are compared with those measured at about 10 °C in the presence of three different guanidine hydrochloride concentrations. At first glance, guanidine hydrochloride seems to cause changes in the PS II reaction pattern that are qualitatively similar to those induced at elevated temperatures. Likewise, guanidine hydrochloride also causes an increase in the effective acceptor pool size in PS II membrane fragments and inside-out vesicles (data not shown), without significantly reducing the number of functionally competent oxygen-evolving complexes. At the low concentrations used in this study, the modifications induced by guanidine hydrochloride

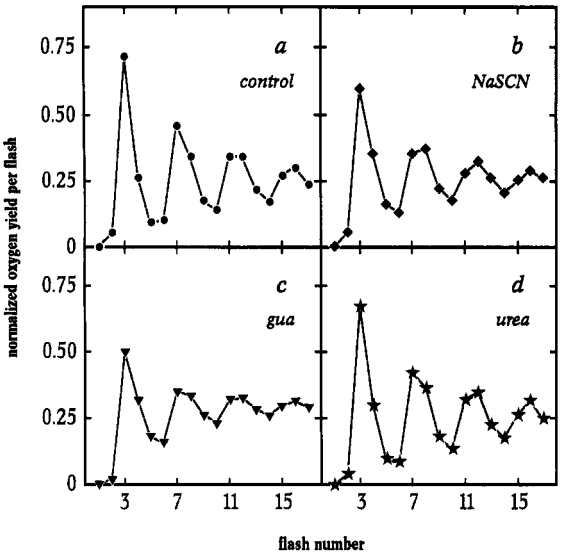

FIGURE 5: Normalized oxygen yield as a function of flash number in dark-adapted spinach thylakoids (no preflash treatment) illuminated by a train of single-turnover flashes at 20 °C and pH 7.6 (20 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tricine). Before the measurements the thylakoids were incubated at 1 mg Chl/mL for 1 h on ice in flow buffer with the following additions: (a) nothing; (b) 100 mM NaSCN; (c) 100 mM guanidine hydrochloride; (d) 400 mM urea. The flow buffer in the Joliot-type electrode does not contain these additions. Other conditions: 2-Hz flash frequency, 3-min incubation time, and −750-mV polarization.

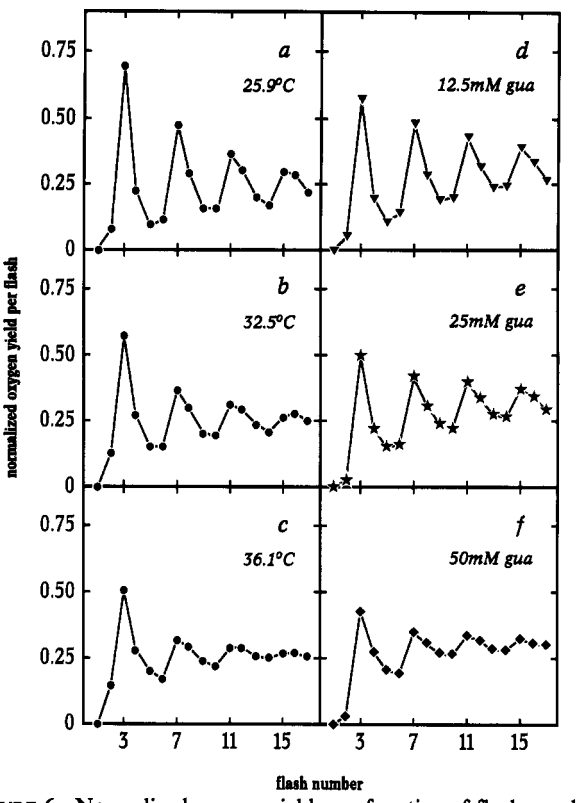

FIGURE 6: Normalized oxygen yield as a function of flash number in dark-adapted $Y_D^{ox}(S_1)$ spinach thylakoids illuminated by a train of single-turnover flashes and measured at different temperatures (left side) or in the presence of different guanidine hydrochloride concentrations at 10 °C (right side). The flow buffer is the same as in Figure 1, except that pH was 6.3 instead of 6.5 and that guanidine hydrochloride was added to the samples and to the flow buffer in the following concentrations: (a–c) 0, (d) 12.5, (e) 25, and (f) 50 mM. The samples were incubated on the electrode for 3 min at (a) 25.9, (b) 32.5, (c) 36.1, and (d–f) 10.4 °C.

are fully reversible (vide infra; Figure 8). A closer inspection of this data, however, reveals that significant differences exist between the thermally induced and guanidine hydrochloride induced changes: (i) the latter treatment does not affect the extent of the oxygen yield due to the second flash, Y_2, and (ii) the lifetimes of S_2 and S_3 are strongly dependent on temperature but virtually invariant to guanidine hydrochloride treatment (vide infra; Figure 9).

In order to obtain more detailed information, the data were evaluated within the framework of the conventional Kok model (the changes of the S_i state populations during the dark time between the flashes are negligibly small at 2 Hz in Y_D^{ox} (S_1) thylakoids). The results obtained reveal that both elevated temperatures and guanidine hydrochloride cause an increase in α (from 0.09 to 0.07 in traces a and d up to 0.14 and 0.12 in traces c and f, respectively), while β is only affected thermally (from 0.04 in trace a to 0.06 in trace c) and remains virtually constant (about 0.03) in the latter treatment. It is interesting to note that a satisfactory fit at higher temperatures (trace c) and guanidine hydrochloride concentrations (trace f) requires increased apparent populations not only of S_0 (up to 15%) but also of "superreduced" S_i states (S_{-1}, and in case of guanidine hydrochloride also S_{-2}) in the dark-adapted samples. The origin of this effect remains to be clarified.

The reversible effects on the PS II reaction pattern induced either by guanidine hydrochloride or elevated temperatures raise the question about a possible interrelation of the two phenomena. In order to analyze this point, experiments were performed at different guanidine hydrochloride concentrations in the temperature range of 10–30 °C. The ratio Y_4/Y_3 was used to monitor changes of the PS II reaction pattern. Figure 7 shows the results obtained from measurements in isolated thylakoids at different guanidine hydrochloride concentrations as a function of temperature. A very interesting feature is observed. The characteristic temperature, ϑ_c, for the thermally induced modification of the PS II reaction pattern is shifted toward lower values with increasing guanidine hydrochloride

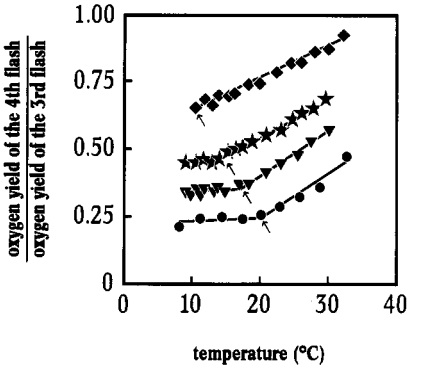

FIGURE 7: Ratio of the oxygen yields of the fourth and third flashes, Y_4/Y_3, as a function of the electrode temperature and the guanidine hydrochloride concentration in dark-adapated $Y_D^{ox}(S_1)$ spinach thylakoids illuminated by a train of single-turnover flashes. Flow buffer and other conditions were the same as in Figure 6: (●) control; (▼) 12.5 mM; (★) 25 mM; (◆) 50 mM.

concentrations. This synergistic type of effect suggests that both the thermally induced and the guanidine hydrochloride induced changes are somehow structurally interrelated, because in the case of independent structural changes guanidine hydrochloride would be anticipated to cause only a constant increment of the Y_4/Y_3 increase without affecting the value of ϑ_c.

In contrast to guanidine hydrochloride, the other chaotropic agents tested give rise to markedly different effects as is illustrated by the following findings: urea (Figure 5d) or Tris (not shown) at even 4-fold concentrations exert only marginal

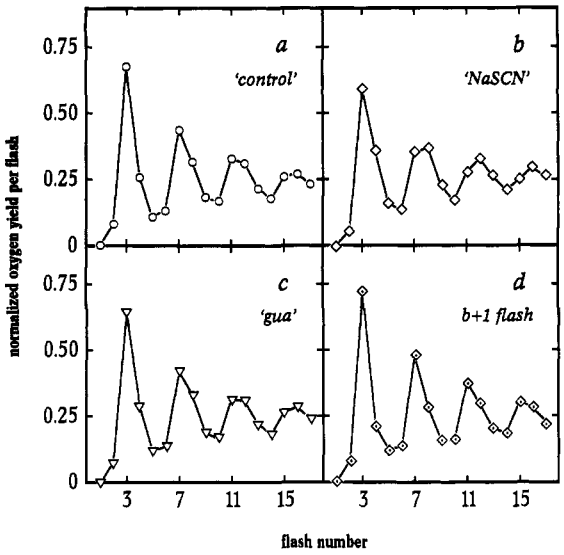

FIGURE 8: Normalized oxygen yield as a function of flash number in dark-adapted spinach thylakoids (no preflash treatment) illuminated by a train of single-turnover flashes at 20 °C and pH 7.6. Before the measurements the thylakoids were incubated at 1 mg Chl/mL for 1 h on ice with the following additions: (a) control without additions; (b and d) 100 mM NaSCN; (c) 100 mM guanidine hydrochloride. After this incubation, the thylakoids were washed twice with flow buffer (0 °C) and were then resuspended to 1 mg Chl/mL. The samples were measured either directly after a 3-min temperature adaptation on the electrode (a–c) or after one preflash and a dark time of 10 min at 20 °C (d). Other conditions were the same as in Figure 5.

(if any) effects on the oscillation pattern of flash-induced O_2 evolution. On the other hand, anions of the Hofmeister series (I^-, SCN^-, and ClO_4^-) induce significant changes, but the underlying mechanism appears to be entirely different from that of the guanidine hydrochloride effect, as shown (for the case of NaSCN) by a comparison of traces b and c in Figure 5. These differences are more clearly illustrated by the data presented in Figure 8. A comparison of traces a and c readily shows that the effect of guanidine hydrochloride can be reversed by removal of this substance in a washing step. In contrast, washing does not eliminate the effect of NaSCN. On the other hand, the change of the oscillation pattern by NaSCN can be easily reversed by a preflash (see trace d of Figure 8). These data suggest that NaSCN probably causes a reduction of Y_D^{ox}.

In order to check this point, the effect of NaSCN on the lifetime of S_2 was measured in thylakoids that were incubated for 1 h on ice with 100 mM NaSCN and subsequently washed twice with buffer in the dark. The results obtained are depicted in Figure 9 (top). It is obvious that NaSCN pretreatment causes an increase of the extent of the fast S_2 decay, while the rate constants of the fast and slow decays remain virtually unaffected. This result is a clear indication that incubation with NaSCN in the dark leads to a decrease of the Y_D^{ox} population. Comparative measurements of the EPR signal II_s confirm the destabilizing effect by NaSCN on Y_D^{ox} (data not shown). In contrast to NaSCN, the addition of guanidine hydrochloride does not exert any effect on the S_2 decay, even if it is not removed by a washing step (Figure 9b).

On the basis of the above findings, it is inferred that chaotropic agents like NaSCN and guanidine hydrochloride cause structural changes in PS II which give rise to significantly different functional effects. NaSCN-type chaotropics seem to destabilize the oxidized form of the redox-active tyrosine Y_D of polypeptide D2 by structural modification of its microenvironment. In order to study this effect in more detail,

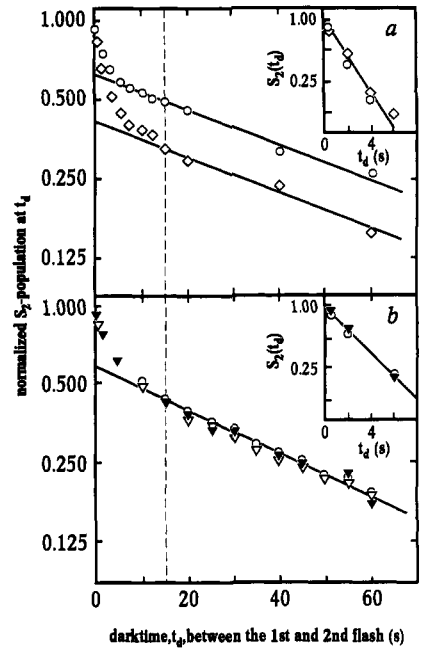

FIGURE 9: Semilogarithmic plot of the normalized S_2 population as a function of the dark time between the first flash and the flash train (2 Hz) in spinach thylakoids (no preflash treatment) at 20 °C and pH 7.6 (20 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tricine). (a, Top): (○) control I; (◇) 1-h incubation with 100 mM NaSCN on ice, two washes with flow buffer. Inset: Fast phase after subtraction of the slow phase and normalization. (b, Bottom): (○) control II; (▼) 1-h incucation with 100 mM guanidine hydrochloride on ice; (▽) same as ▼ but the guanidine hydrochloride was removed from the sample by washing twice with flow buffer before the measurements.

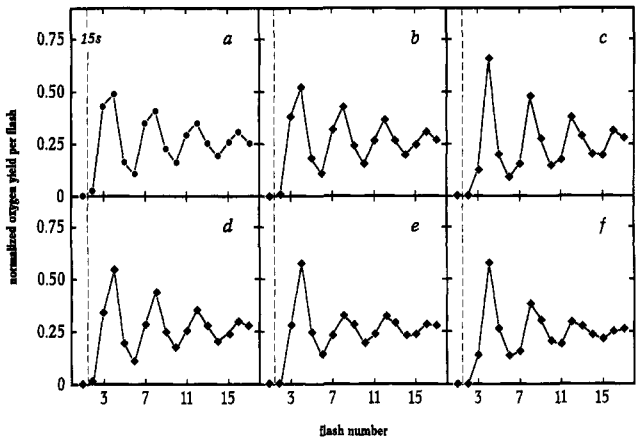

FIGURE 10: Normalized oxygen yield as a function of flash number in dark-adapted spinach thylakoids (no preflash treatment) illuminated with a train of single-turnover flashes at 20 °C and pH 7.6. The dark time between the first flash and the flash train was always 15 s (indicated by - - -). The thylakoids were preincubated on ice for different times and NaSCN concentrations: (a) control; (b) 10 min and 75 mM NaSCN; (c) 4 h and 75 mM NaSCN; (d) 10 min and 150 mM NaSCN; (e) 15 min and 375 mM NaSCN; (f) 15 min and 675 mM NaSCN. Other conditions were the same as in Figure 5.

oscillation patterns of the flash-induced oxygen yield were measured as a function of concentration and dark incubation time with NaSCN. In these experiments, the time between the first and second flashes was kept constant at 15 s, because during this dark period the fast decay of S_2 due to reduction by Y_D is almost complete (see the vertical broken line in Figure 9). Typical results obtained under different incubation conditions are summarized in Figure 10. These data show that the normalized oxygen yield of the 4th flash increases with both the dark incubation time and the NaSCN con-

Table III: Calculated Normalized Y_D^{ox} Population in Thylakoids Incubated in the Dark on Ice in the Absence or Presence of Different Salts with Anions of the Hofmeister Series

salt (0.4 M)	incubation time (h)	$[Y_D^{ox}]$ (%)	salt (0.4 M)	incubation time (h)	$[Y_D^{ox}]$ (%)
no addition	1	0.65	NaBr	3	0.63
$LiClO_4$	1	0.09	NaCl	3	0.62
NaSCN	1	0.15	Na_2SO_4	3	0.60
NaI	1	0.34			

centration. As the rate constants of the fast and slow S_2 decays are virtually invariant with NaSCN (see Figure 9a), oscillation patterns of the type presented in Figure 10 can be used to calculate the population of Y_D^{ox} as a function of incubation time at different NaSCN concentrations. The decline of Y_D^{ox} during dark incubation in the presence of NaSCN can be satisfactorily described by monoexponential kinetics (data not shown). Furthermore, the dark decay of Y_D^{ox} in the absence of NaSCN was determined to be very slow (approximately 2 days, i.e., $k \approx 4 \times 10^{-6}$ s^{-1}) under the incubation conditions (0 °C, pH 7.6). Therefore, this contribution to the overall decay in the presence of NaSCN can be ignored. With these approximations, the concentration dependence of the NaSCN-induced Y_D^{ox} decay was shown to fit a linear relation of the form:

$$k_{NaSCN}(Y_D^{ox}) = k^0_{NaSCN}(Y_D^{ox})[NaSCN] \quad (2)$$

where $k^0_{NaSCN}(Y_D^{ox}) = (1.6 \pm 0.6) \times 10^{-3}$ M^{-1} s^{-1} and [NaSCN] represents the NaSCN concentration.

In general, NaSCN is a redox-active component which could directly interact with Y_D^{ox}. Therefore, the question arises as to whether the NaSCN-induced destabilization of Y_D^{ox} reflects a structural modification due to the chaotropic action or a direct redox interaction. To clarify this point, other anions of the Hofmeister series (Hofmeister, 1888; Collins & Washabough, 1985) were tested for their effects on Y_D^{ox}. Oscillation patterns were measured on samples preincubated in the dark on ice with $LiClO_4$, NaSCN, NaI, NaBr, NaCl, and Na_2SO_4 at concentrations of 0.4 M. An evaluation of the results obtained (data not shown) reveals remarkable differences in the efficiency to destabilize Y_D^{ox}, as is illustrated by calculated Y_D^{ox} populations summarized in Table III. On the basis of these data, the following efficiency sequence can be derived: $LiClO_4$ > NaSCN > NaI ≫ NaBr, NaCl, Na_2SO_4. This series of the anions corresponds well with their positions within the extended Hofmeister series (Collins & Washabough, 1985) but is not related to their redox properties. Therefore, the destabilization of Y_D^{ox} is inferred to be caused by structural changes due to the chaotropic effects of the anions.

DISCUSSION

The present study is an attempt to unravel structure–function interrelations of water cleavage in the PS II complex by analyzing the characteristic period four oscillation pattern of flash-induced oxygen evolution as a function of temperature and effects elicited by chaotropic agents. In general, two problems were addressed: (a) effects on the advancement of the redox states S_i of the water oxidase and (b) effects on the interaction of the water oxidase with the redox-active tyrosine residue Y_D of polypeptide D2 and with other endogenous reductants.

S_i State Advancement. Within the framework of the Kok model (Kok et al., 1970), the shape of the damped period four oscillation can be described by three parameters: (i) the misses parameter, α, as a measure of the probability that excitation with a flash does not lead to a stable redox advancement within the water-oxidizing complex; (ii) the average probability of double hits, β, giving rise to a double redox advancement $S_i \rightarrow S_{i+2}$; and (iii) the initial S_i state population before the flash train.

In general, the phenomenologically determined parameter α can be considered as the composite of two contributions:

$$\alpha_n(\vartheta) = \alpha_{n,o}(\vartheta) + \alpha_n(\vartheta, t_d) \quad (3)$$

where n symbolizes the dependence of the probability of misses on the flash number n of the train, $\alpha_{n,o}(\vartheta)$ is the probability that at temperature ϑ the nth flash does not give rise to a stable charge separation as the prerequisite of the S_i state redox advancement, and $\alpha_n(\vartheta, t_d)$ summarizes effects due to temperature-dependent S_2 and S_3 decays during the dark time t_d between the flashes (the rather slow S_0 oxidation by Y_D^{ox} can be ignored at the flash frequencies used in this study).

The value of $\alpha_{n,o}(\vartheta)$ is determined by redox equilibria at the donor and acceptor sides, giving rise to population probabilities of $P680^+$ and Q_A^- before each flash which depend on the redox states S_i of the water oxidase and of Q_B (Q_B^-), respectively (Renger & Hanssum, 1988; Shinkarev & Wraight, 1993). For the sake of simplicity, a dependence of $\alpha_{n,o}(\vartheta)$ on the flash number n in the train will be ignored, in line with the conventional Kok model, i.e., $\alpha_{n,o}(\vartheta) = \alpha_o(\vartheta)$ for all n. This approximation seems to be justified for contributions due to the acceptor side because no special treatment was performed to obtain a high Q_B population in the dark-adapted samples [for a more detailed discussion of this point, see Rutherford et al. (1984)]. The donor side effects are very difficult to quantify due to a lack of precise data. Therefore, a complicated fitting procedure with a number of assumptions on the redox equilibria of the donor side is not expected to provide further information. A numerical analysis of the experimental data with a kinetic Kok model (see Materials and Methods) using the data of Table I reveals that the contribution $\alpha_n(\vartheta, t_d)$ cannot be the dominating factor for the increase of $\alpha_n(\vartheta)$ above ϑ_c. On the basis of the above-mentioned considerations and approximations (i.e., $\alpha_n(\vartheta) \approx \alpha_o(\vartheta)$; see eq 3), a decrease in the probability of flash-induced stable charge separation is inferred to be responsible for the increase in $\alpha_o(\vartheta)$ at temperatures above ϑ_c. Basically, two different types of modifications can account for this effect: (i) a structural change in the PS II complex and/or its lipidic environment induced at ϑ_c could affect the thermodynamics and/or kinetics, e.g., through changes of distance and/or microenvironment of redox groups; or (ii) $\alpha(\vartheta)_o$ simply reflects the van't Hoff and/or Arrhenius-type temperature dependence of the equilibrium and/or rate constants, respectively, of reactions that determine the value of $\alpha_o(\vartheta)$ (as will be outlined below for the example of the temperature dependence of β). Effects i and ii are difficult to quantify because of the complexity of contributions affecting the value of α (Renger & Hanssum, 1988; Shinkarev & Wraight, 1993). Regardless of this problem, it seems possible that the increase of $\alpha_o(\vartheta)$ above ϑ_c is indicative of a structural change at ϑ_c, because guanidine hydrochloride also gives rise to a similar increase in α and in apparent S_0 and S_{-1} populations without exerting van't Hoff and/or Arrhenius-type changes. Therefore, it appears to be worth considering structural effects briefly.

Different lines of evidence have been reported in the literature for thermally induced structural changes in thylakoids. It was found that the activation energy for the dark decay of membrane energization exhibits a breakpoint near

20 °C (Kraayenhof et al., 1971; Gräber & Witt, 1975). Likewise, an analogous discontinuity was found for ADRY agent induced S_2/S_3 decay (Renger, 1975). These phenomena probably reflect structural changes in the lipid bulk phase of the thylakoid membrane although more localized effects in protein complexes cannot be entirely excluded. Analogously, the temperature dependence of the period four oscillation pattern in algal cells and intact chloroplasts (Maisson & Lavorel, 1977; Delrieu, 1978) might not be exclusively due to changes within PS II but could also comprise modifications due to indirect effects such as membrane destacking, protein phosphorylation, etc.

As an attempt to separate bulk membrane effects from those that are more closely related to PS II, experiments were performed using three types of preparations with different membrane organizations: thylakoids, PS II membrane fragments, and detergent-free PS II-enriched inside-out vesicles. All three preparations exhibited nearly the same temperature dependence of Y_4/Y_3 (see Figure 2a) as an indirect measure of α (see Figure 2d). It therefore seems reasonable to assume that this thermal effect is mainly restricted to changes in the PS II complex and/or its lipidic microenvironment. With respect to an increase of α induced by structural changes, only the redox equilibrium $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$ will be considered because virtually no information is available on donor side effects. Different lines of evidence suggest that a structurally induced shift at the acceptor side is responsible for the temperature-independent increment toward higher values of Y_4/Y_3 in PS II membrane fragments compared with thylakoids and inside-out vesicles; modifications near the Q_B site have been observed in PS II membrane fragments as reflected by changes in atrazine binding (Renger et al., 1986) and the kinetics of Q_A^- reoxidation (Haag et al., 1992). Furthermore, removal of Triton X-100 from PS II membrane fragments by treatment with Bio-Beads markedly reduces the difference from thylakoids and inside-out vesicles. An analogous shift of the redox equilibrium could be responsible for the thermally induced increase of $\alpha_o(\vartheta)$ to above ϑ_c. The nature of reversible structural changes possibly induced at elevated temperatures and their influence on the value of α remain to be clarified.

Another consequence of thermal activation is the increase of the double hit probability β. It was shown that electron transfer to Q_A is indispensible for sufficient stabilization of $P680^+$ in order to permit water oxidation (Renger & Eckert, 1980). Therefore, β is a measure of a sequential double turnover at Q_A, which acts as univalent electron acceptor under normal conditions (Witt, 1973). Excitation with flashes of a few microseconds duration is expected to give rise to β values which nearly linearly correlate with the rate constant k_2 of Q_A^- reoxidation by $Q_B(Q_B^-)$ or exogenous electron acceptors (see the Appendix). Accordingly, the temperature dependence of β should correspond with that of k_2. A semilogarithmic plot of β as a function of reciprocal temperature (data from Figure 2e) exhibits a nearly linear relation with a slope corresponding to an activation energy of about 30 kJ/mol (data not shown). This value nicely fits with recent results obtained for the activation energy of Q_A^- reoxidation by Q_B in thylakoids (Renger et al., 1993), thereby demonstrating that under our excitation conditions the value of β is determined by k_2. Correspondingly, the β values are markedly smaller in PS II membrane fragments, as expected from the slower Q_A^- reoxidation compared to thylakoids (Haag et al., 1992).

Interaction of the Water Oxidase with Y_D and the Acceptor Side. The redox advancement of the S_i states in the water oxidase takes place via univalent electron abstraction steps with Y_Z^{ox} as the direct oxidant. It was shown that the reaction coordinates of the processes described by eq 1 exhibit a characteristic pattern of activation energies with strong dependences on the redox state S_i in PS II preparations from cyanobacteria (Koike et al., 1987) and higher plants (Renger & Hanssum, 1992). In marked contrast, the data of this study reveal that the interaction of the water oxidase with the redox-active tyrosine Y_D of polypeptide D2 is not only slower by many orders of magnitude but also the temperature dependence is quite different. The most striking phenomenon is the rather small difference in the activation energies of S_2 and S_3 reduction by Y_D [this study; see also Vass et al. (1990)], whereas the corresponding values of the oxidative forward reaction of S_1 (to S_2) and S_2 (to S_3) with Y_Z^{ox} differ by a factor of about 3 (Koike et al., 1987; Renger & Hanssum, 1992). Although the exothermicity of the reductive pathway with Y_D significantly exceeds that of the oxidative reactions with Y_Z^{ox} [about 170 versus 40–50 mV: Vass and Styring (1991) and Vos et al. (1991), respectively], the activation energies should markedly differ for the fast phase decays of S_2 and S_3 if the reorganization energies are taken into account which were recently reported for the transitions $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ (Renger & Hanssum, 1992). This expectation relies on the assumption that the redox group(s) of the water oxidase in the oxidation states S_2 and S_3 becomes predominantly reduced by direct electron transfer from Y_D. This condition, however, does not seem to be satisfied if one takes into account a recent analysis on electron transport in biological systems (Moser et al., 1992).

Within the framework of a simple nonadiabatic description given by Fermi's golden rule, the rate constant of electron transport is given by the expression

$$k_{ET} = \frac{2\pi}{\hbar}|V_R|^2 FC \tag{4a}$$

where V_R is the quantum mechanical matrix element coupling reactant and product electronic wave functions at a distance R of the interacting redox groups and FC symbolizes the Franck–Condon weighted density of states. As the electronic wave functions exponentially decay in space, $|V_R|^2$ can be phenomenologically described by

$$|V_R|^2 = |V_o|^2 \exp(-\beta R) \tag{4b}$$

where V_o is the matrix element for the case of redox groups being in direct van der Waals contact.

On the basis of a thorough analysis of different redox systems, Moser et al. (1992) deduced a β value of 14 nm^{-1} for biological systems. It now seems well established that the manganese cluster of the water oxidase is closer to Y_Z (distance R_Z) than to Y_D (distance R_D). Although only limited information is available from the literature (Hoganson & Babcock, 1988; Evelo et al., 1989), it seems reasonable to assume that the difference between distances R_D and R_Z is not significantly smaller than 1.5 nm. On the basis of this value, the optimum rate constants of electron transfer between the redox-active tyrosines and the water oxidase should differ by at least a factor on the order of 10^9. Accordingly, the direct reduction of S_2 and S_3 by Y_D is expected to take place in the time domain of hours or days. This range exceeds that of the experimentally well-established value of a few seconds (see also Table I) by several orders of magnitude. The rough estimation reveals that, with a probability of nearly 100%, the electron transport pathway from Y_D to the water oxidase in S_2 and S_3 comprises additional redox-active groups. The most likely candidates for this function are the couples $P680/P680^+$

and Y_Z/Y_Z^{ox}. Therefore, the electron transfer from S_2 and S_3 is inferred to be highly dominated by the indirect pathway via Y_Z/Y_Z^{ox} and P680/P680$^+$. This conclusion, based on simple considerations within the framework of electron-transfer theory, is supported by independent lines of evidence [Buser et al., 1992; see also Vass and Styring (1991)].

The slow phase reduction of S_2 and S_3 by the PS II acceptor side and other endogenous donors requires even higher activation energies. These processes certainly comprise the participation of various intermediates and therefore will not be discussed in more detail.

Effects of Chaotropic Agents. A phenomenon that appears to be of special interest is the remarkably different mode of action among the class of substances referred to as chaotropic agents. These compounds are known to modify the structure of proteins at sufficiently high concentrations by changing the interaction preferences among the constituents within the ternary system of solvent–protein–solute [Timasheff (1992) and references cited therein]. At high solute concentrations, this effect probably causes the elimination of the oxygen evolution capacity (Homann, 1992). At the lower concentrations used in this study, however, the chaotropic agents induce specific modifications of the PS II complex. In this respect, generally three types of chaotropic agents can be distinguished: (a) urea and Tris are without significant effect; (b) guanidine hydrochloride enhances the probability of misses and of superreduced states of the water oxidase in a manner comparable to thermal activation; and (c) anions of the Hofmeister series (e.g., SCN^-, ClO_4^-, I^-) destabilize the oxidized form of the redox-active tyrosine Y_D of polypeptide D2.

These specific effects are very likely the consequence of different selective structural changes. Therefore, it appears worthwhile to compare common properties of compounds belonging to groups b and c. At first glance, the electric charge is the most striking difference between type b and type c substances. It is therefore tempting to speculate that positive versus negative charge is mainly responsible for the different structural effects. If this is really the case, the net charges of the target protein domains will be different in both cases. This idea would provide a reasonable explanation for the action of the anions of the Hofmeister series. As the microenvironment of Y_D^{ox} appears to be positive, these negative species should be attracted. This interaction probably induces structural modifications giving rise to a reduced barrier of the hydrophobic shield around Y_D, deduced from model studies (Svensson et al., 1990; Ruffle et al., 1992), so that Y_D^{ox} with its rather high oxidation potential of about 750 mV (Boussac & Etienne, 1984) can attain the redox equilibrium with the surrounding medium much faster. In marked contrast to the structural changes caused by anions of the Hofmeister series, guanidine hydrochloride leads to modifications which enhance the probability of losses in the process of stable radical pair formation. Interestingly, guanidine hydrochloride also enhances the accessibility of PS II to dithiothreitol (DTE) (Irrgang et al., 1992). Whether these two phenomena are intimately interrelated is a topic of future investigations. The present study shows that chaotropic agents at suitable concentrations provide a useful tool to induce specific structural changes of functional relevance in the PS II complex.

APPENDIX

Calculation of Double Hit Probability β. As the oxidizing redox equivalents produced at P680$^+$ can only be used for water oxidation if a stable charge separation is achieved by electron transfer to Q_A (Renger & Eckert, 1980), the reoxidation rate of Q_A^- determines the β value at saturating flash intensities. The differential recovery dQ_A within the time interval between t and $t + dt$ is given by

$$d[Q_A] = -d[Q_A^-] = k_2 e^{-k_2 t}\, dt \qquad (A1)$$

where $[Q_A^-]$ is the concentration of Q_A^- at time t normalized to $[Q_A^-]_o = 1$ and k_2 is the rate constant of Q_A^- reoxidation.

The probability of a double hit $d\beta$ within dt at time t can be calculated by

$$d\beta = \Phi(\varphi,k_2)\sigma\varphi(t)k_2 e^{-k_2 t}\, dt \qquad (A2)$$

where $\Phi(\varphi,k_2)$ is the quantum yield of a second turnover of Q_A, σ is the optical cross-section of PS II, and $\varphi(t)$ is the photon density of the flash at time t. Accordingly, the double hit probability is obtained by integration of eq A2.

$$\beta = k_2 \int \Phi(\varphi,k_2)\sigma\varphi(t)e^{-k_2 t}\, dt \qquad (A3)$$

Under our flash excitation conditions (Xenon flashes with FWHM of 3 μs; see Materials and Methods), the integral is only weakly dependent on k_2 and can be considered to be constant in a first-order approximation. Then β is linearly related to k_2.

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