Biochimica et Biophysica Acta 850 (1986) 173–183
Elsevier

BBA 42009

Studies on the nature of the water-oxidizing enzyme. II. On the functional connection between the reaction-center complex and the water-oxidizing enzyme system Y in Photosystem II

W. Weiss and G. Renger *

Max-Volmer-Institut für Biophysikalische und Physikalische Chemie der Technischen Universität, Strasse des 17. Juni 135, D 1000 Berlin 12 (Germany)

(Received October 22nd, 1985)

Key words: Photosystem II; Water splitting; Trypsin; Difference absorption spectrometry; P-680; Reaction center

Laser-flash-induced absorption changes have been measured with a time resolution of 1–2 μs in PS II membrane fragments. The following was found. (1) In the presence of 0.5 mM K_3 [$Fe(CN)_6$] at pH = 6.0 absorption changes at 830 nm induced by repetitive flash excitation in PS II membrane fragments pretreated (10 min) with trypsin at pH = 7.5 are dominated by 10–15 μs kinetics. Under the same conditions the relaxation kinetics at 325 nm are slower by at least two orders of magnitude. (2) In the same samples μs components contributing to the overall kinetics of transient ultraviolet absorption changes have the same half-life time as the 830 nm relaxation kinetics within the range of $4.0 \leq pH \leq 8.0$. (3) At pH = 6.0 the extent of 10–15 μs kinetics of ultraviolet absorption changes as a function of wavelength is characterized by peaks at 260 nm, 300 nm, 360 nm (positive) and 340 nm (negative). It is vanishingly small in the range of 315–325 nm. (4) In the presence of 1 mM hydroxylamine dark-adapted samples illuminated with a few flashes ($n \leq 8$) revealed the same pattern at 270 nm and 830 nm as PS II membrane fragments trypsinized at pH = 7.5. In contrast to that, after preillumination with 70 flashes the absorption changes at 270 nm and 830 nm exhibit slower kinetics in the microsecond range with half-life times of 200–300 μs. These results are interpreted as a light requiring disconnection of Z from P-680 by hydroxylamine. (5) After subtraction of the difference spectrum for the reduction of the primary plastoquinone acceptor Q_A the extent of the separated initial amplitudes (limited by time resolution of 1–2 μs) as a function of wavelength exhibit characteristic features, depending on the functional integrity of the water-oxidizing enzyme system Y. If the samples were deprived of their oxygen-evolving capacity the data obtained resemble the difference spectrum reported for oxidation of chlorophyll *a* in solution (Borg, D.C., Fajer, J., Felton, R.H. and Dolphin, D. (1970) Proc. Natl. Acad. Sci. USA 67, 813–820). On the other hand, a markedly different spectrum is obtained in samples with a functionally competent water-oxidizing enzyme system Y. Based on kinetical arguments this difference spectrum is inferred to reflect Z-oxidation in oxygen-evolving PS II membrane fragments. The shape of the Z^{ox}/Z difference spectrum appears to be slightly affected by the functional integrity of systems Y. (6) No direct evidence was obtained for the existence of a kinetically and spectrally distinguishable redox component D_x between Z and P-680 in samples that were deprived of their oxygen-evolving capacity. On the

* To whom correspondence should be addressed.

Abbreviations: Chl *a*, chlorophyll-*a*; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; $D_{1,2}$, redox components connecting P-680 and the water-oxidizing enzyme system Y; PS II, Photosystem II; Ph, pheophytin; P-680, photoactive chlorophyll of Photosystem II; Q_A, primary plastoquinone acceptor of Photosystem II; Z, Photosystem II donor component of which the oxidized form gives rise to EPR-signal II_f and II_{vf}.

basis of the present data the mode of functional coupling between system Y and P-680 is discussed. Furthermore, implications of possible changes in the electrostatic interactions between the redox active chromophors and the surrounding protein matrices are considered.

Introduction

Despite of intensive research activities many details of the functional organization of photosynthetic water oxidation are not yet clarified (for a recent review, see Ref. 1). Generally, three basic mechanistic questions have to be answered in order to understand the overall process. (a) Which arrangement of a photoactive pigment and coupled redox groups within the reaction center complex is required for high quantum yield generation of redox equivalents with an oxidizing power sufficient for water oxidation? (b) What is the molecular mechanism of dioxygen formation at the catalytic center of the water-oxidizing enzyme system Y? (c) What is the coupling mechanism between the photooxidized chlorophyll *a* group, P-680$^+$, of the reaction-center complex and system Y? The present study deals with problems related to the third question.

Kinetic measurements revealed that the reaction-center complex and system Y are functionally coupled via at least one redox component, referred to as D_1 or Z. Its oxidized from Z^{ox} exhibits a characteristic EPR-spectrum [2]. Selective destruction of the water-oxidizing enzyme system Y does not affect the flash-induced formation of this EPR-signal [3]. Accordingly, the elimination of the oxygen-evolving capacity by appropriate treatments does not block the electron transfer from Z to P-680$^+$, but the kinetics of P-680$^+$-reduction were found to become retarded significantly [4]. Likewise, the relaxation kinetics of the flash-induced EPR signal changed markedly. Accordingly, these signals are referred to as signal II_{vf} (system Y functionally intact) and signal II_f [2,3]. The slowing down of P-680$^+$-reduction after destruction of system Y permitted comparative studies with the formation kinetics of signal II_f (these emasurements were just at the limit of the time resolution of the EPR equipment). It was shown, that both kinetics almost perfectly coincide, so that Z was inferred to be electronically coupled directly to P-680 [5]. Furthermore, the relaxation kinetics of the EPR signal II_{vf} observed in samples that are fully active in oxygen evolution [6] closely resemble those for the redox state transitions in the water-oxidizing enzyme system Y [7,8]. Therefore these kinetic data could favour the idea that Z^{ox} is the only detectable redox component which functionally connects system Y with the reaction-center complex. On the other hand, considerations about the P-680$^+$-reduction kinetics suggest the possible participation of a further redox component D_x [9,10]. However, so far no direct evidence has been presented for the existence of D_x. A comparison of the shape of signal II with the spectra of model substances led to the conclusion that the functional group of Z in its oxidized state represents a plastosemiquinone cation radical [11]. This assignment requires a specific protein matrix to keep the protonated form sufficiently stable [12] and implies that the observed proton release due to Z^{ox} formation [13,14] does not arise directly from the redox active prosthetic group of Z.

Recently the Z^{ox}/Z difference spectra in the ultraviolet range were reported [12,15,16] for samples with selectively destroyed system Y. These spectra are in line with the idea that the functional group of Z^{ox} is a special plastosemiquinone cation radical. However, it should be mentioned that these data were obtained from measurements with restricted time resolution. Furthermore no spectra are reported for Z^{ox}/Z under conditions of fully intact system Y. In this study laser-flash-induced absorption changes were measured in order to obtain information about: (a) the Z^{ox}/Z-difference spectrum under conditions of system Y being functionally intact, (b) possible effects of the destruction of system Y on the Z^{ox}/Z-difference spectrum and (c) the possible participation of further redox components in the functional coupling between the catalytic site in system Y and the reaction-center complex.

Materials and Methods

PS II membrane fragments with high oxygen-evolving capacity were prepared from market spinach as described in Ref. 17, with some modifications outlined in Ref. 18. Mild trypsin treatment was performed by incubation of PS II in the dark at a 1 : 1 trypsin/chlorophyll w/w-ratio. Trypsin was purchased from Boehringer (Mannheim). For a selective modification of the acceptor side trypsination in the dark was performed at pH < 6.5 (for 5–10 min) and the samples were immediately used after the dark treatment. Measuring times up to 10 min did not diminish the activity by more than 10%. Complete inhibition of the oxygen-evolving capacity was achieved by trypsination in the dark at pH = 7.5 (Chl/trypsin = 1 : 1). After trypsination the samples were diluted by a factor of 20 and adjusted to the desired pH-values, at which the measurements were performed.

The additions to suspensions used for the experiments are indicated in the figure legends. Absorption changes in the ultraviolet region were performed by a conventional flash spectrophotometer with a pulsed measuring light beam as described in Ref. 19. Laser flash excitation occurred 1 ms after opening the shutter for the detecting beam (illumination time per measuring light pulse, 5 ms; intensity, approx. 100 $\mu W/cm^2$; optical bandwidth, approx. 4 nm). Absorption changes at 830 nm were measured in a similar way as in Ref. 20, but with a markedly improved time resolution and sensitivity. All measurements were performed at an optical pathlength of 1 cm. The samples were excited with pulses from a frequency-doubled Nd-Yag-laser (5–7 ns full width at half maximum (FWHM); $\lambda = 532$ nm).

Absolute oxygen yield measurements under repetitive flash excitation (Xenon flashes, 10–15 μs FWHM) were performed with a Clark-type electrode as outlined in Ref. 21.

Results

Laser-flash-induced absorption changes in PS II membrane fragments that were deprived of their oxygen evolving capacity

In order to eliminate interfering effects due to reactions of system I and to improve the optical properties of the samples the measurements were performed with PS II-membrane fragments that exhibit negligibly small PS I-activity. These preparations also permit to analyze easily effects due to destruction of the water-oxidizing enzyme system Y, because it was shown, that trypsination at pH = 7.5 completely destroys the oxygen-evolving capacity, whereas the same procedure at pH = 6.0 leaves this activity almost unaffected [18,20].

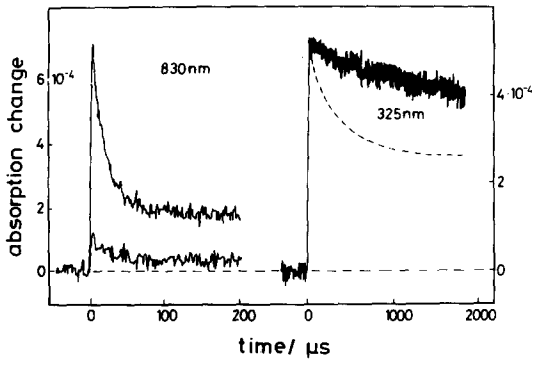

Fig. 1. Absorption changes at 830 nm and 325 nm as a function of time in trypsin incubated (pH 7.5) PS II particles measured at pH 6.0. The suspension contained: PS II-particles trypsinized at pH = 7.5 (30 μM chlorophyll)/10 mM NaCl/20 mM Mes-NaOH (pH = 6.0). The small signal at the left side is measured without an exogeneous acceptor, the other signals were obtained with 0.5 mM K_3 [$Fe(CN)_6$]. Addition of 10 mM $CaCl_2$ accelerates the relaxation kinetics at 325 nm (dashed line), whereas the kinetics at 830 nm remain unchanged (not shown). Time between the flashes 0.7 s. 16 and 32 signals were averaged for measurements at 830 nm and 325 nm, respectively. Other conditions as described in Materials and Methods.

Fig. 1 shows absorption changes induced at 830 nm and 325 nm by repetitive laser flash excitation in PS-II-membrane fragments that were trypsinized at pH = 7.5. These absorption changes reflect the turnover of P-680 and predominantly of the primary plastoquinone acceptor Q_A, respectively, within the reaction-center complex. In the absence of exogenous acceptors only a small absorption change is observed, due to blockage of Q_A^- reoxidation by Q_B in trypsinized membranes [22,23]. In the presence of $K_3[Fe(CN)_6]$ the relaxation of the 830 nm absorption change is dominated by a 10–15 μs kinetics, whereas the decay at 325 nm is slower by orders of magnitudes. These findings correspond with previous

results obtained in inside-out vesicles, that were trypsinized at pH = 7.4 [20]. The data show, that P-680$^+$ becomes mainly reduced by Z in trypsinized (pH = 7.5) PS II membrane fragments. Accordingly, between the flashes an almost complete Z regeneration has to occur. This implies that in contrast to Tris-washed thylakoids [24,25] and PS II membrane fragments obtained by the use of CHAPS/digitonin the back reaction between Q_A^- and P-680$^+$ contributes only marginally to P-680$^+$ reduction, because the electron transfer from Z to P-680$^+$ (10–15 μs) is much faster than that from Q_A^- to P-680$^+$ (150–300 μs).

Based on these kinetical properties one would expect that the back reaction could contribute to not more than 10% of the overall relaxation kinetics at 830 nm, provided that Z becomes completely regenerated between the flashes. Accordingly, the slow component at 830 nm observed in Fig. 1 might be due to a small fraction of PS II that undergoes back reaction between Q_A^- and P-680$^+$. If this would be really the case, one might expect to elicit the back reaction $Z^{ox} \cdot$ P-680$^+$ Q_A^- $\rightarrow Z^{ox} \cdot$ P-680 Q_A if Z^{ox} is kept oxidized by decreasing the time between the flashes. To clarify this point, double-flash experiments were performed. It was found that even at a dark time t_d of 1 ms between the 1st and 2nd flash of each group the extent of the slow kinetics of absorption changes caused by the 2nd flash remains unaffected, whereas the extent of the 10–15 μs kinetics decreased markedly. The extrapolated initial amplitude (at a time resolution of approx. 1 μs the back reaction between P-680$^+$ and Ph^- cannot be observed) of the 10–15 μs kinetics due to the 2nd flash depends on the time t_d between the two flashes. The half maximum is achieved at $t_d \approx 15$ ms (data not shown). This result indicates the in the majority of PS II the reduction of Z^{ox} appears to be faster than Q_A^- reoxidation, i.e., the latter process determines the extent of the overall reaction ($t_{1/2} = 10$–15 ms under our conditions). A simple explanation for these findings could be offered by the assumption of a heterogeneity of PS II in our samples with Z^{ox} reduction being rather slow in a small fraction (approx. 25%) giving rise to back reaction between Q_A^- and P-680$^+$, whereas in the majority of PS II Z^{ox} reduction is faster than Q_A^- reoxidation. Further experiments are required to test this idea. For the problems that will be attacked in this paper the origin of the slow relaxation component at 830 nm is not relevant. It is interesting to note that in the presence of 10 mM $CaCl_2$ the relaxation kinetics of the 325 nm absorption changes become accelerated (dashed curve in Fig. 1, right side), whereas the 830 nm decay kinetics remain unaffected (data not shown). This result indicates that $CaCl_2$ also influences reactions at the acceptor side in Triton X-100 fractioned PS II membranes. This idea is supported by the latest findings that reveal a remarkable $CaCl_2$ effect on the PS II acceptor side as reflected by herbicide binding and the efficiency of *p*-benzoquinone as exogenous electron acceptor (Renger, G., Fromme, R. and Hagemann, R., unpublished results). The underlying mechanism for this $CaCl_2$ effect has to be clarified. Regardless of these details PS II membrane fragments trypsinized at pH = 7.5 are shown to provide proper samples in order to analyze the functional connection between P-680 and Z and to determine the difference spectrum of Z^{ox}/Z by a method that differs from the techniques which were used in previous studies [12,15,16]. If Z directly reduces P-680$^+$, then the extent of the 10–15 μs-kinetics reflects the extinction coefficient difference $\Delta\epsilon_{Z^{ox}/Z}$ minus $\Delta\epsilon_{P\text{-}680^+/P\text{-}680}$. Furthermore,

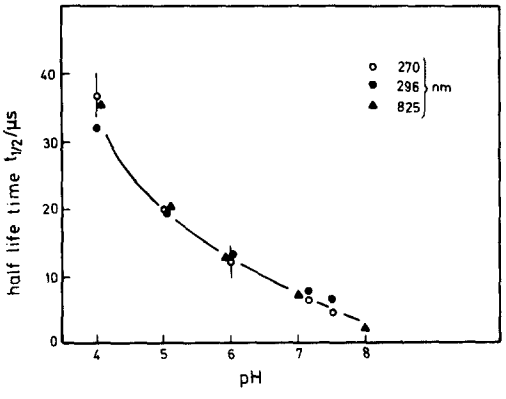

Fig. 2. Half-life time of absorption changes at 270 nm, 296 nm and 830 nm in trypsinized (pH = 7.5) PS-II particles as a function of pH. The suspension composition was the same as in Fig. 1, except of the buffer and that 10 mM $CaCl_2$ was used in all experiments. The buffers at 20 mM were: succinate at pH = 4.0, Mes-NaOH at $5.0 \leq$ pH ≤ 6.5, Hepes at pH = 7.0 and Tricine-NaOH at $7.2 \leq$ pH ≤ 8.0.

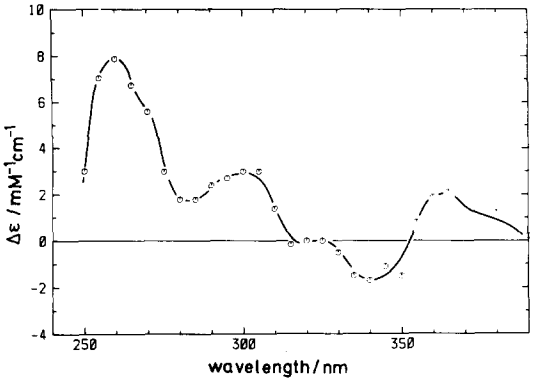

Fig. 3. Extent of the 10–15 μs-kinetics as a function of wavelength in trypsinized (pH = 7.5) PS II particles. Experimental conditions as in Fig. 1.

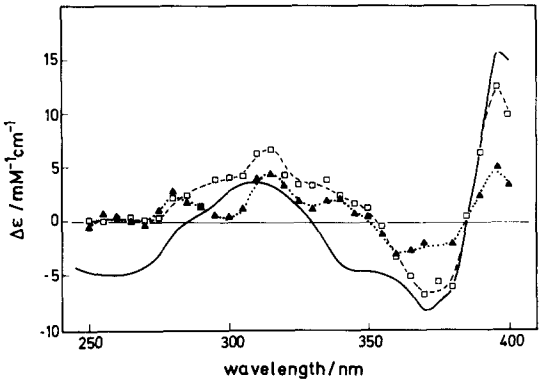

Fig. 4. Separated difference spectra of P-680$^+$/P-680 (dotted and dashed curve) and of chlorophyll a^+/chlorophyll a (continuous line) according to Borg et al. (1970). The P-680$^+$/P-680-difference spectra in trypsinized (pH = 7.5) (▲) and in NH_2OH-treated (pH = 6.0) PS-II particles (□) measured at pH = 6.0 were obtained by a procedure that is described in the text. Experimental conditions as described in Material and Methods.

these kinetics should reveal the characteristic pH dependence of the half-life time that was observed in normal thylakoids [4] and inside out vesicles [20]. The data obtained for absorption changes at 830 nm (reflecting P-680$^+$-reduction) and at 296 nm and 270 nm (indicating predominantly Z^{ox}-formation), that are depicted in Fig. 2, reveal this to be the case.

The extent of the 10–15 μs kinetics (measured at pH = 6.0) was obtained by using a semilogarithmic plot of the data that was extrapolated to $t = 0$. The results are depicted in Fig. 3 as a function of wavelength. Surprisingly, the shape of this function resembles remarkably the Z^{ox}/Z spectrum reported previously by different groups [12,15,16]. This might indicate that the P-680$^+$/P-680 difference spectrum in the ultraviolet region exhibits rather small extinctions in the range of 250–380 nm, in contrast to what was found for Chl a^+/Chl a in solution [26].

Determination of the difference spectrum for P-680-oxidation in the ultraviolet region

In order to clarify this point we attempted to determine the P-680$^+$/P-680 difference spectrum in the ultraviolet region in trypsinized PS II-membrane fragments. As P-680$^+$ becomes reduced mainly via a 10–15 μs kinetics (at pH = 6.0) in PS II membrane fragments, trypsinized at pH = 7.5, the extent of the extrapolated initial amplitude (kinetically unresolved at a 1–2 μs time resolution) as a function of wavelength represents the difference spectra $\Delta\epsilon_{\text{P-680}^+/\text{P-680}}$ plus $\Delta\epsilon_{Q_A^-/Q_A}$. Accordingly after subtraction of the well-known $\Delta\epsilon_{Q_A^-/Q_A}$ spectrum the difference spectrum of $\Delta\epsilon_{\text{P-680}^+/\text{P-680}}$ is obtained. On the basis of a number of 300 Chl/reaction center (determined in control samples by measurements of the average

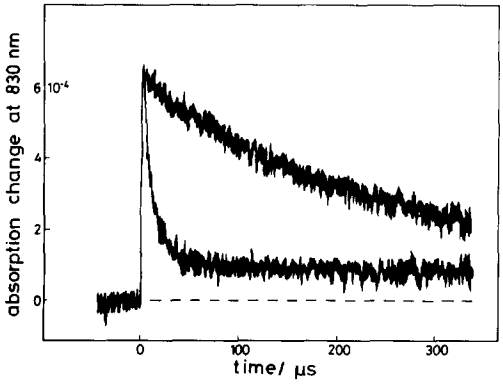

Fig. 5. Absorption changes in NH_2OH incubated PS-II-particles at 830 nm as a function of time after a laser flash. The fast relaxation kinetics represents the average of absorption changes induced by flashes 1–8 in dark-adapted samples. After preillumination with 70 flashes signals with slow relaxation kinetics were obtained by averaging eight traces. The suspension contained: PS II-particles (30 μM chlorophyll), 0.5 mM $K_3[Fe(CN)_6]$, 1 mM NH_2OH, 10 mM NaCl and 20 mM Mes-NaOH. Time between laser flashes, 0.7 s.

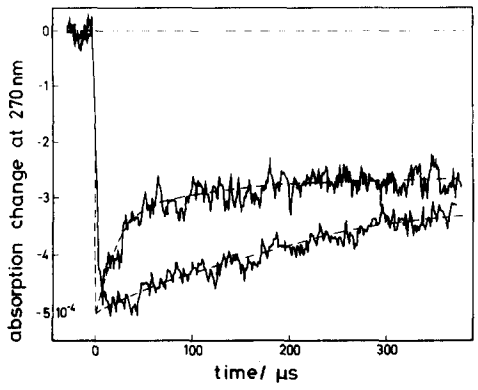

Fig. 6. Absorption change at 270 nm as a function of time in PS-II particles at pH = 6.0. Top curve: PS-II particles, trypsinized at pH 7.5; lower curve: PS-II particles incubated with 1 mM NH_2OH, addition of 10 mM $CaCl_2$. The measurements were performed with 0.5 mM $K_3[(Fe(CN)_6]$. Time between laser flashes, 0.7 s; 256 signals were averaged.

oxygen yield per flash) the data depicted in Fig. 4 (filled triangles) are obtained.

Another method that can be applied is the measurement of absorption changes in samples where Z becomes functionally disconnected from P-680. This has been reported to be the case if samples are illuminated in the presence of hydroxylamine [27]. Figs. 5 and 6 show absorption changes at 270 nm and 830 nm observed at pH = 6.0 in the presence of 1 mM NH_2OH in PS II-membrane fragments that were not trypsinized.

If the samples were illuminated with only a few laser flashes, the donor function of Z remains fully intact as reflected by the 10–15 µs relaxation kinetics at 830 nm (indicating P-680$^+$ reduction) and 270 nm (predominantly due to Z^{ox} formation), but the oxygen-evolving capacity is destroyed. In contrast to that, after preillumination with more than 50 flashes, the relaxation completely changes to a kinetics with 200–300 µs. This kinetics is indicative for a back reaction between P-680$^+$ and Q_A^- under the above-mentioned conditions. Therefore, the extent of the kinetically unresolved fast phase of the absorption changes as a function of wavelength should again reflect the sum of $\Delta\epsilon_{P\text{-}680^+/P\text{-}680}$ plus $\Delta\epsilon_{Q_A^-/Q_A}$. Taking into account the number of reaction centers (1 per 300 chlorophylls) and the data for $\Delta\epsilon_{Q_A^-/Q_A}$ from the literature [28], the data described in Fig. 4 (open squares) are obtained. Both methods result in spectra with a generally similar shape in the range 250–400 nm. However, remarkable differences are observed around 300 nm and above 360 nm. The origin of these differences is not yet clarified. It seems likely that they reflect – at least partly – changes in the microenvironment of P-680 exerted by different treatments of the samples. A comparison with in vitro data for $\Delta\epsilon_{Chl\ a^+/Chl\ a}$ in CH_2Cl_2 solution reveals similarities in the range 280–400 nm. The small negative peak of the Chl a^+/Chl a difference spectrum at 250–260 nm and negative values between 330 and 355 nm are not observed.

Determination of the difference spectrum for Z-oxidation in the ultraviolet region and the dependence of the spectral shape on the functional integrity of the water-oxidizing enzyme system Y

$\Delta\epsilon_{P\text{-}680^+/P\text{-}680}$ now permits the determination of the Z^{ox}/Z-difference spectrum from the data of Fig. 3. In order to correct the results obtained with the same sample type, the values of $\Delta\epsilon_{P\text{-}680^+/P\text{-}680}$ in Fig. 4, that are represented by closed triangles, were used. The results obtained are shown in Fig. 7 (open squares). Generally, the same result for $\Delta\epsilon_{Z^{ox}/Z}$ should be found by using the amplitudes remaining approx. 100 µs after the flash and subtracting the difference spectrum $\Delta\epsilon_{Q_A^-/Q_A}$. This was found to be the case (data not shown). However, this procedure does not provide information about $\Delta\epsilon_{P\text{-}680^+/P\text{-}680}$. A comparison of the Z^{ox}/Z-difference spectrum obtained in this study with previously reported data (for comparison, the spectrum of Ref. 16 is redrawn in Fig. 7 as a dashed curve) exhibits deviations that could be due to the use of different sample preparations. In order to check this point, absorption changes at 325 nm were measured in Tris-washed PS II membrane fragments under conditions (time resolution, 250 ns) that exclude masking effects due to limited time resolution of the P-680$^+$ reduction kinetics. The results depicted in Fig. 8 indicate that decay kinetics of 10–15 µs is not observed (after 1 µs a small transient increase (up to 5% of the total amplitude) rather than a decrease could be resolved). If $\Delta\epsilon_{Z^{ox}/Z}$ would be really vanishingly small, P-680$^+$ formation should not give rise to positive absorption changes at 325 nm, in contrast to what was found in this study for

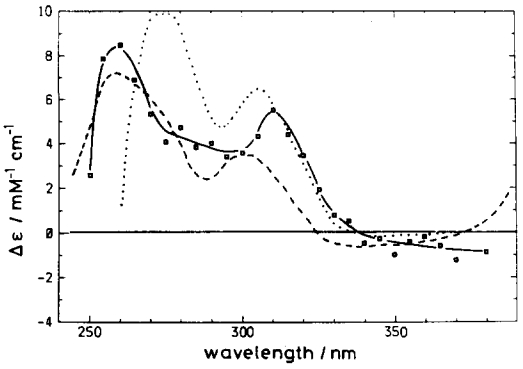

Fig. 7. Separated difference spectrum of Z^{ox}/Z. The details of the calculations are described in the text. For comparison the Z^{+}/Z-spectra of Dekker et al. [16] (dashed curve) and the difference spectrum of durosemiquinone cation radical/duroquinol (dotted curve) of Land (see Ref. 16) are depicted.

$\Delta\epsilon_{P\text{-}680^{+}/P\text{-}680}$ as well as for $\Delta\epsilon_{Chl\ a^{+}/Chl\ a}$ in solutions at 325 nm [26]. On the basis of these considerations we conclude that $\Delta\epsilon_{Z^{ox}/Z}$ at 325 nm is not negligible. A comparison of the calculated Z^{ox}/Z-difference spectrum with data reported for the difference spectrum of the durosemiquinone cation radical formation in solution reveals similarities between both spectra. (Fig. 7, dotted curve).

The main deviation appears to be a 15 nm hypsochromic shift of the 280 nm band and a slight bathochromic shift (up to 5 nm) of the band peaking around 305 nm. If one accepts that these shifts are caused by differences in the microenvironment of the chromophore, the data of Fig. 7 are in line with the assumption that the prosthetic redox group in Z^{ox} is a specifically bound plastosemiquinone cation radical [11]. The data in Fig. 2 suggest, that Z directly reduces $P\text{-}680^{+}$. However, these measurements were performed with a limited time resolution of 1–2 μs. Under these conditions lag phases in the 10–15 μs kinetics were observed at some wavelengths (e.g., at 270 nm, data not shown). The appearance of lag phases could be indicative for a reaction sequence that implies the participation of a further redox component D_x. Theoretical considerations show that lag phases can arise even without the existence of D_x in the simple P-680-Z-redox system at wavelength regions of large negative $\Delta\epsilon_{Q_A^{-}/Q_A}$ and positive $\Delta\epsilon_{Z^{ox}/Z}$ values (and vice versa), if the measurements are limited by a microsecond time resolution of the equipment. In order to eliminate these effects experiments were performed at 270 nm with a time resolution of 250 ns. At this wavelength region the effect giving rise to 'artificial' lag phases should be especially pronounced. It was found that lag phases observed at 270 nm with a time resolution of 1–2 μs are totally absent when the same experiments were performed with a time resolution of 250 ns. Therefore, the present data do not provide direct evidence for the existence of a further kinetically and spectroscopically distinguishable redox component D_x. The results also show that a sufficiently high time resolution is required in order to avoid kinetical artifacts. The spectral deviation of $\Delta\epsilon_{P\text{-}680^{+}/P\text{-}680}$ observed in differently treated samples suggest that microenvironmental effects could play an important role regarding the shape of the difference spectra. Therefore, the question arises about the possible influence of the destruction of the water-oxidizing enzyme system Y on the Z^{ox}/Z-difference spectrum. So far, this spectrum has not been reported for samples with intact system Y. In this case the set of absorption changes is much more complex, because of the significantly faster electron transfer to $P\text{-}680^{+}$ [29–31] and the subsequent reactions between Z^{ox} and the water-oxidizing enzyme system Y. The following attempt to determine the Z^{ox} difference spectrum is based on the most simple scheme for the donor side that implies P-680, Z and system Y. If one takes into account the electron transfer kinetics (for $P\text{-}680^{+}$-reduction [29–31] and the $S_i \rightarrow S_{i+1}$ redox transitions [6–8], the amplitude of absorption changes measured 2 μs after the excitation laser flashes should reflect mainly the sum of the difference spectra of $\Delta\epsilon_{Q_A^{-}/Q}$ plus $\Delta\epsilon_{Z^{ox}/Z}$. Accordingly, $\Delta\epsilon_{Z^{ox}/Z}$ is obtained as the difference between the measured 'initial' amplitude at 2 μs and $\Delta\epsilon_{Q_A^{-}/Q_A}$.

Dark adapted PS II-membrane fragments trypsinized at pH = 6.0 were illuminated in the presence of 10 mM $CaCl_2$ and 0.5 mM $K_3[Fe(CN)_6]$ as exogenous acceptor (under these conditions system Y remains fully active in oxygen evolution; see Renger G., Fromme, R. and Hagemann, R., unpublished data). Trypsin treatment at pH = 6.0 was used in order to eliminate effects due to surface exposed polypeptides that could be modified at pH = 6.0 and at pH = 7.5. In the

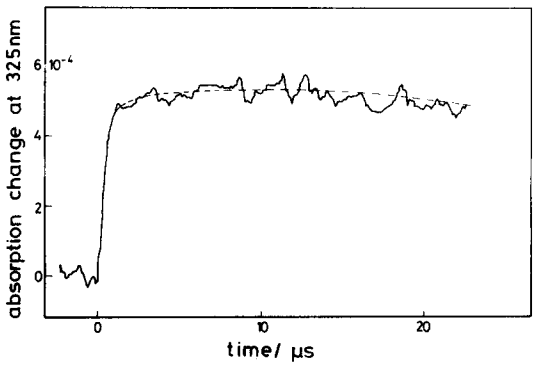

Fig. 8. Absorption change at 325 nm in Tris-washed PS-II particles as a function of time after the laser flash. The suspension contained: 10 μM chlorophyll, 0.4 mM $K_3[Fe(CN)_6]$, 10 mM NaCl, 20 mM MES/NaOH, pH 6.0. Time between the flashes, 0.7 s. 512 signals were averaged.

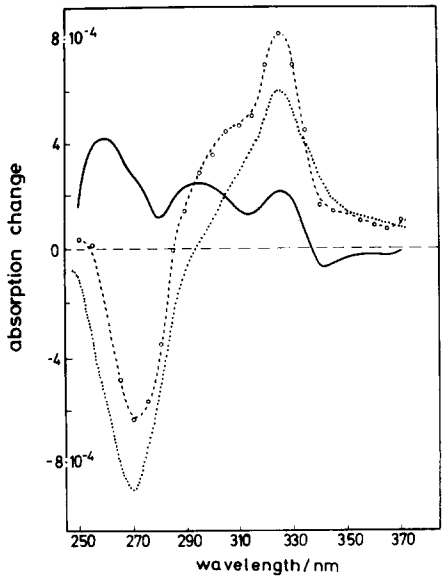

Fig. 9. Average extent of the apparent initial amplitude as a function of wavelength in dark adapted PS II particles (dashed curve). The values of the absorption changes induced by the 2nd, 3rd and 4th flash were averaged. The suspension contained PS-II particles (15 μM chlorophyll) 15 μg/ml Trypsin, 10 mM $CaCl_2$, 10 mM NaCl and 20 mM Mes-NaOH, pH = 6.0. Dotted curve: Q_A^-/Q_a-difference spectrum (see Ref. 28); full curve: difference between the dashed curve and the Q_A^-/Q_A difference spectrum (dotted curve).

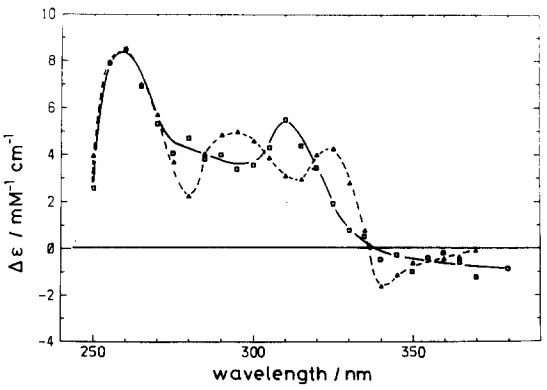

Fig. 10. Z^{ox}/Z-difference spectra in PS-II particles with full competent oxygen-evolving capacity (▲) and of completely OEC-deprived samples. (■) The data are redrawn from Fig. 7 and 9.

presence of $K_3[Fe(CN)_6]$, the 2-μs amplitude of the absorption changes due to the first flash was markedly smaller than in the following flashes where the amplitudes are almost constant. In Fig. 9 the average values of the amplitudes at 2 μs of absorption changes caused by the 2nd to 4th flash are depicted as a function of the wavelength. The data obtained are dominated by the contribution of the difference spectrum $\Delta\epsilon_{Q_A^-/Q_A}$. For the determination of $\Delta\epsilon_{Z^{ox}/Z}$ the absolute number of intact photosystems II is required. This value was determined to be one active system per 300 chlorophylls by measurements of the average oxygen yield per flash with a Clark type electrode. With the use of this value and taking the spectrum of $\Delta\epsilon_{Q_A^-/Q_A}$ from the literature [28] (dotted curve in Fig. 9), the spectrum of $\Delta\epsilon_{Z^{ox}/Z}$ represented by the full curve is obtained. In order to be able to compare this spectrum with that of Z^{ox}/Z in samples that were completely deprived of their oxygen-evolving capacity, the data of Figs. 7 and 9 were redrawn in Fig. 10. The spectra inhibit an excellent correspondence of the main band peaking around 260 nm. In the wavelength region between 275 nm and 340 nm systematic deviations are observed between the Z^{ox}/Z-difference spectra of samples with intact and destroyed water-oxidizing enzyme system Y, respectively. These deviations are probably not due to uncertainties caused by the limitations of the applied procedure, because they exhibit alternating regions of positive and negative differences between the spectra.

Discussion

The results presented in this study reveal that the shape of the separated difference spectrum $\Delta\epsilon_{Z^{ox}/Z}$ depends on the functional integrity of the water-oxidizing enzyme system Y (see Fig. 10). Likewise, in PS II membrane fragments that were deprived of their oxygen-evolving capacity $\Delta\epsilon_{P\text{-}680^+/P\text{-}680}$ exhibit differences if system Y is destroyed either by trypsination at pH = 7.5, or by incubation with hydroxylamine (see Fig. 4). As all the difference spectra $\Delta\epsilon_{Z^{ox}/Z}$ and $\Delta\epsilon_{P\text{-}680^+/P\text{-}680}$ were obtained by subtraction of $\Delta\epsilon_{Q_A^-/Q_A}$ taken from the literature [28] two alternative explanations can be offered for the observed deviations: (a) if different sample treatments applied in this study would affect $\Delta\epsilon_{Q_A^-/Q_A}$ the spectral differences could be simply due to changes of $\Delta\epsilon_{Q_A^-/Q_A}$; (b) if $\Delta\epsilon_{Q_A^-/Q_A}$ remains invariant, the data of Figs. 4 and 10 really reflect environmental effects that modify $\Delta\epsilon_{Z^{ox}/Z}$ and $\Delta\epsilon_{P\text{-}680^+/P\text{-}680}$. A change of $\Delta\epsilon_{Q_A^-/Q_A}$ seems to be less likely for the following reasons: (1) a modification of the donor side is expected to affect the functional chromophores of P-680 and Z more severely than Q_A at the acceptor side; and (2) mild trypsination at pH = 6.0 that markedly reduces herbicide binding at the PS II acceptor side does not change the EPR-signal of $Q_A^- \cdot Fe^{2+}$, indicating an almost unaltered microenvironment of Q_A [32].

Of course, this finding does not unambiguously exclude possible spectral changes of $\Delta\epsilon_{Q_A^-/Q_A}$ in the ultraviolet range. Despite of lacking a direct proof we still conclude that effects due to changes of $\Delta\epsilon_{Q_A^-/Q_A}$ play a minor role for the phenomena observed in Figs. 4 and 10. Based on this assumption changes of the polypeptide structure at the PS II donor side caused by trypsination [20] or NH_2OH-treatment [33] are inferred to affect the difference spectra $\Delta\epsilon_{P\text{-}680^+/P\text{-}680}$ and $\Delta\epsilon_{Z^{ox}/Z}$ predominantly. It should be kept in mind that in NH_2OH-treated samples preilluminated by 70 flashes redox component Z becomes functionally disconnected from P-680, whereas trypsin treatment at pH = 7.5 does not interrupt electron transfer from Z to P-680$^+$. Therefore, the microenvironment of Z is expected to be modified in a different way. It appears reasonable to assume that this effect also influences the difference spectrum $\Delta\epsilon_{P\text{-}680^+/P\text{-}680}$. The underlying molecular mechanism remains to be clarified. In the case of $\Delta\epsilon_{Z^{ox}/Z}$, the alternating positive and negative deviations caused by the destruction of the water-oxidizing enzyme system Y favour the idea of changes in local electrochromic effects. It has been concluded that the negative charge of Q_A^- causes an electrochromic band shift of pheophytin that gives rise to the C550 absorption changes [34]. Likewise, the rearrangement and/or removal of point charges in the proteinaceous environment due to destruction of the water-oxidizing enzyme system could lead to electronic band shifts of Z and Z^{ox} that cause the observed changes of $\Delta\epsilon_{Z^{ox}/Z}$.

The possibility of spectral changes due to modifications of the polypeptide pattern at PS II rises another interesting question: to what extent are affected the spectral properties of chromophores acting as prosthetic groups by detergents that are used for the isolation of PS II membrane fragments? Further experiments are required to clarify this point.

Changes in the microenvironment of redox-active chromophores, however, can not only affect their spectral properties, but also induce significant redox potential shifts. A well-known phenomenon in PS II is the modification of the cytochrome *b*-559-apoprotein, that leads to transformation of the high potential into the low potential form [35]. Accordingly, analogous effects could be relevant for other prosthetic groups, especially for P-680$^+$/P-680 and Z^{ox}/Z. It has been discussed that the destruction of the water-oxidizing enzyme system Y shifts the redox potential of Z^{ox}/Z towards less positive values [9] thereby changing the electron-transfer kinetics from Z to P-680$^+$ [36]. Recently, in Tris-washed thylakoids this redoxpotential shift of Z^{ox}/Z was estimated to be approx. 120 mV [37]. An alternative explanation, however, could be offered by the assumption that P-680 and Z are connected by a further redox component D_x and that Tris washing enhances the redox potential of D_x^{ox}/D_x relative to that of P-680$^+$/P-680 [38]. In this case the equilibrium P-680$^+$ $D_x \rightleftharpoons$ P-680 D_x^{ox} could be shifted towards the left and D_x^{ox} would escape the detection as a spectroscopically and kinetically resolvable redox state. This assumption would also

be in line with the finding that in Tris-washed chloroplasts the donor capacity beyond P-680 amounts to only one electron per PS II [39]. Likewise, the failure to obtain direct evidence for the existence of D_x in the present study could be explained by the assumption that destruction of the water-oxidizing enzyme system Y leads to the above-mentioned equilibrium shift. However, it has to be emphasized, that our data do not permit an unambiguous conclusion about the possible existence of D_x because theoretical calculation shows that lag phases in the kinetics of absorption changes could be masked in a system containing three reactants, provided that the extinction coefficients of the participating reactants have appropriate values (data not shown).

The existence of two redox components in the electron-transport pathway from the catalytic site of the water-oxidizing enzyme system Y to $P\text{-}680^+$ has been postulated on the basis of indirect kinetical evidence [9]. Later, this scheme was used in order to explain the nanosecond relaxation kinetics of 830 nm absorption changes that were observed in oxygen evolving systems [10]. To test whether D_x exists as a kinetically and spectroscopically distinguishable species if the water oxidizing enzyme system Y remains intact, but escapes the detection after system Y destruction, further experiments are required with a much higher time resolution. Experiments are in progress to solve this problem.

Acknowledgements

The authors would like to thank M. Völker for the PS II membrane fragments, B. Hanssum for reading the manuscript and A. Schulze for drawing the figures. The financial support by Deutsche Forschungsgemeinschaft (SFB 312) is gratefully acknowledged.

References

1 Renger, G. and Govindjee (1985) Photosynth. Res. 6, 33–57
2 Babcock, G.T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 315–328
3 Yocum, C.F., Yerkes, C.T., Blankenship, R.E., Sharp, R.R. and Babcock, G.T. (1981) Proc. Natl. Acad. Sci. USA 78, 7507–7511
4 Conjeaud, H. and Mathis, P. (1980) Biochim. Biophys. Acta 590, 353–359
5 Boska, M., Sauer, K., Buttner, W. and Babcock, G.T. (1983) Biochim. Biophys. Acta 722, 327–330
6 Babcock, G.T., Blankenship, R.E. and Sauer, K. (1976) FEBS Lett. 61, 286–289
7 Dekker, J.P., Plijter, J.J., Ouwehand, L. and Van Gorkum, H.J. (1984) Biochim. Biophys. Acta 767, 176–179
8 Renger, G. and Weiss, W. (1985) Biochem. Soc. Trans. 14, 17–20
9 Gläser, M., Wolff, C. and Renger, G. (1976) Z. Naturforsch. 31c, 712–721
10 Brettel, K., Schlodder, E. and Witt, H.T. (1984) Biochim. Biophys. Acta 766, 403–514
11 O'Malley, P.J., Babcock, G.T. and Prince, R.C. (1984) Biochim. Biophys. Acta 766, 283–288
12 Weiss, W. and Renger, G. (1984) FEBS Lett. 169, 219–223
13 Förster, V. and Junge, W. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II, pp. 305–308, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
14 Renger, G. and Völker, M. (1982) FEBS Lett. 149, 203–207
15 Diner, B. and Vitry, C. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 407–412, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
16 Dekker, J.P., Brok, M. and Van Gorkom, H.J. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 171–174, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
17 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234
18 Völker, M., Ono, T., Inoue, Y. and Renger, G. (1985) Biochim. Biophys. Acta 806, 25–34
19 Renger, G. and Weiss, W. (1983) Biochim. Biophys. Acta 722, 1–11
20 Renger, G., Völker, M. and Weiss, W. (1984) Biochim. Biophys. Acta 766, 582–591
21 Renger, G. (1972) Biochim. Biophys. Acta 256, 428–439
22 Renger, G. (1976) Biochim. Biophys. Acta 440, 287–300
23 Renger, G. and Weiss, W. (1982) FEBS Lett. 137, 217–221
24 Renger, G. and Wolff, C. (1976) Biochim. Biophys. Acta 423, 610–614
25 Haveman, J. and Mathis, P. (1976) Biochim. Biophys. Acta 440, 346–355
26 Borg, D.C., Fajer, J., Felton, R.H. and Dolphin, D. (1970) Proc. Natl. Acad. Sci. USA 67, 813–820
27 Ghanotakis, D.F. and Babcock, G.T. (1983) FEBS Lett. 153, 231–234
28 Dekker, J.P. (1985) Thesis, Rijksuniversiteit Leiden
29 Van Best, J.A. and Mathis, P. (1978) Biochim. Biophys. Acta 408, 154–1163
30 Brettel, K. and Witt, H.T. (1983) Photobiochem. Photobiophys. Acta 408, 154–1163
31 Eckert, H.J., Renger, G. and Witt, H.T. (1984) FEBS Lett. 167, 316–320
32 Renger, G., Rutherford, A.W. and Völker, M. (1985) FEBS Lett. 185, 243–247
33 Tamura, N. and Cheniae, G. (1985) Biochim. Biophys. Acta 809, 245–259
34 Van Gorkum, H. (1976) Thesis, Rijksuniversiteit, Leiden

35 Butler, W.L. and Matsuda, H. (1983) in The Oxygen-Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, M. Renger, G. and Satoh, K., eds.), pp. 113–122, Academic Press Japan, Tokyo
36 Renger, G., Eckert, H.J. and Weiss, W. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, M., Renger, G. and Satoh, K., eds.), pp. 73–82, Academic Press Japan, Tokyo
37 Yerkes, C.T., Babcock, G.T. and Crofts, A.R. (1983) FEBS Lett. 158, 359–363
38 Van Gorkom, H.J. (1985) Photosynth. Res. 9, 97–112
39 Coujeaud, H., Mathis, P. and Paillotin, H. (1979) Biochim. Biophys. Acta 546, 280–291