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SPECTRAL CHARACTERIZATION IN THE ULTRAVIOLET REGION OF THE PRECURSOR OF PHOTOSYNTHETICALLY EVOLVED OXYGEN IN ISOLATED TRYPSINIZED CHLOROPLASTS

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The reaction mechanism of the water-splitting enzyme system Y has been analyzed by measurements of ultraviolet absorption changes induced by a flash train in dark-adapted chloroplasts. In order to eliminate processes which gives rise to interfering absorption changes with a binary oscillation, mildly trypsizined chloroplasts were applied which are known to be disconnected from the secondary plastoquinone acceptor B, but are able to evolve oxygen, provided that $K_3Fe(CN)_6$ is used as electron acceptor (Renger, G. (1976) Biochim. Biophys. Acta 440, 287–300). It was found that: (a) The extent of the relaxation kinetics of the 320 nm absorption change accomplished within 5 ms oscillates with a periodicity of four in phase with the oxygen yield as a function of the number of the flash in the train. (b) The difference spectra of the difference between the initial amplitudes and those remaining after a 4.5 ms dark period, referred to as $\Delta A_n^{4.5}$, exhibit pronounced bands peaking around 320 nm (positive) and 270 nm (negative). The extent oscillates in the flash train. It is at a maximum for the absorption change differences induced by the third flash, $\Delta A_3^{4.5}$. (c) Taking into account the fact that the first flash of the train does not yield oxygen, whereas the oxygen production is at a maximum due to the third flash, the difference of $\Delta A_3^{4.5} - \Delta A_1^{4.5} = \Delta A_{3,1}^{4.5}$ as a function of wavelength is interpreted as the difference spectrum of the precursor of molecular oxygen in system Y. The spectrum exhibits a band peaking around 320 nm. Accordingly, the precursor is referred to as Y-320. The data obtained are discussed in terms of a molecular model proposed for the mechanism of photosynthetic water oxidation (Renger, G. (1977) FEBS Lett. 81, 223–228).

Introduction

The reaction pattern of Photosystem II which leads to photolytic water cleavage by visible light is characterized by the intermediary accumulation of oxidizing and reducing equivalents at the donor and acceptor sides. This gives rise to oscillations of the redox states with periodicities of four and two, respectively (for a review see Ref. 1). It is well known that the redox reaction sequence at the acceptor side involves special plastoquinone molecules as functional groups which are embedded in a functional protein matrix, referred to as X320-B apoprotein [2]. The water oxidation to molecular oxygen by oxidizing redox equivalents generated by the photoreaction of Chl a_{II} was found to occur through a four-step univalent mechanism which can be best described by the S_i-state 'clock' of Kok et al. [3]. Despite the fact that different lines of

Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; ANT-2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; ANT-2s, 2-(3,4,5-trichloro)anilino-3,5-dinitrothiophene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 2-(*N*-morpholino)ethanesulfonate; Chl, chlorophyll; DMSO, dimethyl sulfoxide; PQ, plastoquinone.

evidence suggest manganese playing a pivotal role as functional center of the water-splitting enzyme system Y, an unambiguous substantiation of the S_i states is still lacking (for a review see Ref. 4 and 5). In order to explain the rather high stability of the state S_i, participation of a redox couple (symbolized by M/M^+), which does not necessarily contain manganese as active functional group, has been proposed [6]. Theoretical considerations about the energetics of photosynthetic water oxidation suggest that cytochrome *b*-559 could be a likely candidate for the M/M^+ couple [4,6]. However, as yet there is no experimental evidence to support the substantiation of the M/M^+ couple as being cytochrome *b*-559. Another component which was claimed to be involved in the reaction sequence of water oxidation to molecular oxygen is plastoquinone [7,8]. This conclusion coincides with recent findings indicating that the oscillation pattern of ultraviolet absorption changes in the 290–370 nm range, induced by a train of short, saturating flashes in dark-adapted chloroplasts, appears to be a superposition of two different oscillations with periodicities of two and four, respectively [9,10]. The latest findings of Velthuys [11] corroborate these results. Since a separation of the reaction sequences of the donor and acceptor sides could not be achieved in the same sample, the difference spectrum of the absorption changes in normal and hydroxylamine-treated chloroplasts was claimed to reflect the characteristics of the donor side in normal chloroplasts. However, as Pulles et al. [9] inferred that at least two types of absorption changes contribute to the oscillation pattern in hydroxylamine-treated chloroplasts and that about 30% of B stays reduced (B^-) in dark-adapted chloroplasts, it remains an unresolved problem whether the differences of absorption changes detected in normal and hydroxylamine-treated chloroplasts are solely due to the turnover of the S_i states. Therefore, another independent procedure appears to be desirable for the analysis of the reaction pattern of the donor side of Photosystem II in normal chloroplasts.

Based on measurements of absorption changes at 334 nm and oxygen evolution, trypsin was inferred to disconnect the primary plastoquinone acceptor of Photosystem II (X320) from the secondary plastoquinone acceptor, B. At the same time, a highly increased accessibility of $X320^-$ towards oxidation by $K_3Fe(CN)_6$ was observed [12,13]. According to this, it should be possible, under carefully selected experimental conditions, to eliminate the binary oscillation of the acceptor side without seriously affecting the water-splitting enzyme system. A first attempt to apply the trypsinization method to the analysis of the spectral properties of system Y was found to be succesful [14]. The relaxation kinetics in the millisecond range of absorption changes at 320 nm in dark-adapted isolated spinach chloroplasts were shown to oscillate with a periodicity of four, resembling those of the flash-induced oxygen evolution. This assignment of the absorption changes to reactions in the water-splitting enzyme system Y is corroborated by the finding that the oscillation pattern becomes suppressed by ADRY reagents which selectively catalyze the decay of S_2 and S_3 states [15,16].

The present paper provides further evidence for the participation of components in the reaction sequence leading the water cleavage, which are characterized by absorption changes in the ultraviolet. An analysis is made based on the S_i-clock model of Kok et al. [3] and the molecular model of Renger [4,6].

Materials and Methods

Isolated Class II spinach chloroplasts were prepared according to the method of Winget et al. [17] except that 10 mM ascorbate was present in the grinding medium and 5% DMSO was added for storage in liquid nitrogen.

The procedure for mild trypsinization is described in Ref. 18. The standard reaction mixture contained chloroplasts (15 μM Chl), 2 mM $MgCl_2$, 10 mM KCl and 20 mM Mes-NaOH, pH 6.5. Further additions were as indicated in the legends to the figures.

Absorption changes were measured by a flash photometer [21] equipped with a pulsed measuring light beam switched on for 30 ms. The intensity of the measuring light was 50 or 100 $\mu W/cm^2$. Theoretical calculations indicate that no more than 10% of the reaction centers of Photosystem II become excited during the detecting light pulse. Experimental data are in line with this estimation, be-

cause a decrease in the measuring light intensity by a factor of two did not alter the observed pattern of the absorption changes within the experimental error of the data.

After each train containing the required number of flashes (usually four flashes), the sample in the cuvette was automatically renewed (via magnetic valves) from a stock suspension of dark-adapted chloroplasts. 16–64 signals were averaged in an NIC 1170 apparatus. Excitation xenon flashes (15 μs half-width, saturating intensity) passed through a Schott RG1 filter. Optical pathlength, 10 mm; optical bandwidth, 2 nm; electrical bandwidth, 200 kHz.

Results

Fig. 1 shows a typical trace of absorption changes at 320 nm induced by a train of four flashes (each separated by a 1 s interval) in dark-adapted trypsinized chloroplasts in the presence of $K_3Fe(CN)_6$ as exogenous electron acceptor. The signals exhibit a characteristic oscillation pattern in their relaxation kinetics. These oscillations are inferred to reflect mainly the turnover of the water-splitting enzyme system Y leading to oxygen release. This conclusion is based on the fact that: (a) the electron transfer from the primary (X320) to the secondary (B) plastoquinone acceptor is interrupted in trypsinized chloroplasts [12] so that binary oscillations due to the acceptor side [19] can be excluded; and (b) the rise kinetics of S_1, S_2 and S_3 are fast enough [25] and the decay kinetics of S_2 and S_3 via deactivation, which are not seriously modified by mild trypsinization [18], are slow enough so that the flash-induced transitions $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ as well as the deactivation of S_2 and S_3 should not contribute to the observed millisecond relaxation.

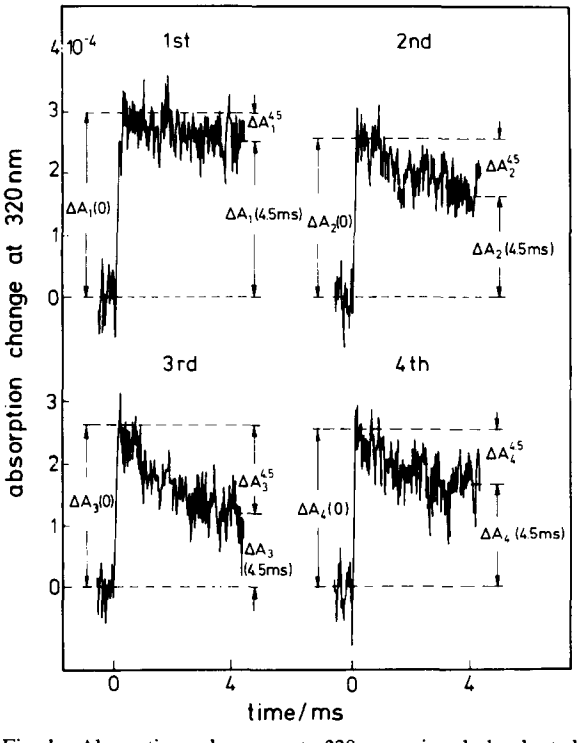

Fig. 1. Absorption changes at 320 nm in dark-adapted trypsinized chloroplasts illuminated by a train of four flashes separated by a dark time of $t_d = 1$ s. Electron acceptor, 0.5 mM $K_3Fe(CN)_6$; trypsin, 15 μg/ml chloroplast suspension; 28 samples were averaged.

As the time resolution of our measurements did not resolve the kinetics of the reactions $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$, the apparent initial amplitude $\Delta A_n(0)$ can be used as an appropriate approximation in order to neglect effects due to these transitions. Our results did not reveal significant 400-μs kinetics which would reflect an $S_2 \rightarrow S_3$ transition [25] so that also possible effects due to this reaction can be of only minor importance. Accordingly, the difference between the amplitudes of the absorption change initially induced by the nth flash, $\Delta A_n(0)$, and those remaining after $t = 4.5$ ms, $A_n(4.5)$, referred to as $\Delta A_n^{4.5} = \Delta A_n(0) - \Delta A_n(4.5$ ms) (see Fig. 1), can be separated into two contributions: (i) an absorption change reflecting the release of oxygen (in terms of the scheme of Kok et al. [3], the transition $S_3 \xrightarrow{h\nu} S_4 \rightarrow S_0 + O_2$) which should oscillate in its amplitude in the same manner as the oxygen evolution; and (ii) absorption changes due to other processes (involving the $X320^-$ reoxidation by external $K_3Fe(CN)_6$) which are expected to be nonoscillating. If one assumes that the latter contribution to $\Delta A_n^{4.5}$ remains invariant in a flash sequence, it is reflected by the absorption change difference due to the first flash, $\Delta A_1^{4.5}$, because no oxygen evolution takes place in this case.

In order to test the above-mentioned considerations and to see if they are reasonable, the effect of

different agents on the absorption changes has been investigated. DCMU hardly affects the lifetimes of S_2 and S_3 [20] in trypsinized chloroplasts. Therefore, the characteristic pattern described in Fig. 1 should not be affected markedly by DCMU. On the other hand, ADRY agents are expected to eliminate the oscillations of the relaxation kinetics because these agents were found to be active also in trypsinized chloroplasts [18]. If $K_3Fe(CN)_6$ as external electron acceptor is substituted by benzyl viologen, $X320^-$ becomes reoxidized by S_2 via an internal electron cycle [14]. Accordingly, in this case no oscillation should occur and the extent of the absorption changes caused by the flash train (except the first one) is determined by the rate of the back-reaction.

The results obtained (DCMU data not shown, other data described in Ref. 14) fully correspond with the interpretation that the oscillatory millisecond relaxation kinetics really do reflect absorption changes at 320 nm which are caused by the formation of oxygen in the water-splitting enzyme system Y.

The oscillation patterns of the oxygen yield and of the millisecond relaxation kinetics of the 320 nm absorption changes, $\Delta A_n^{4.5} - \Delta A_1^{4.5}$, are compared in Fig. 2. The oxygen yield pattern is computed for the initial S_i-state population of $[S_0]_0 = 0.3$ and $[S_1]_0 = 0.7$ and probabilities of misses and double hits being $\alpha = \beta = 0.1$. In order to take into account the effect of the 30 ms measuring light pulse, its average probability of an S_i-state advancement has to be calculated. With the assumption of a Poisson distribution of the photons and averaging over the total sample, an S_i-state advancement probability of $\gamma_{m.b.} = 0.1$ has been estimated. As the millisecond relaxation of the 320 nm absorption change caused by the first flash of the train cannot be due to oxygen evolution, the difference of amplitudes $\Delta A_{n,1}^{4.5} = \Delta A_n^{4.5} - \Delta A_1^{4.5}$ is depicted in Fig. 2. The data, normalized to the extent of the third flash, reveal a fairly close correspondence between the calculated oxygen yield and $\Delta A_{n,1}^{4.5}$. Therefore, they corroborate the conclusion that a component, tentatively referred to as Y-320, is the precursor of the photosynthetically evolved oxygen. For a further characterization of this component, the difference spectra of the absorption changes induced by a train of four

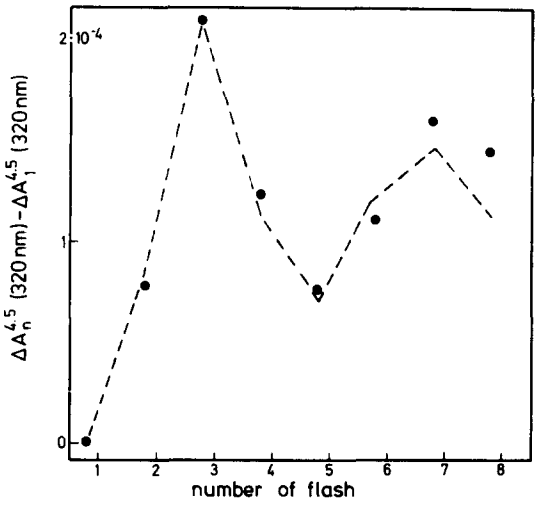

Fig. 2. Difference between the nth and the first flash of the difference of the initial amplitude and that at 4.5 ms after the flash, $\Delta A_{n,1}^{4.5}$ (full circles) and oxygen yield, as a function of the number of the flash in a train. The oxygen yield represented by the broken line has been computed according to the scheme of Kok et al. [3] with $[S_0]_0 = 0.3$, $[S_1]_0 = 0.7$, $[S_2]_0 = [S_3]_0 = 0$, $\alpha = 0.1$, $\beta = 0.1$ and a measuring light pulse parameter of $\gamma_{m.b.} = 0.1$. Experimental conditions as in Fig. 1

flashed in dark-adapted, trypsinized chloroplasts have been measured using $K_3Fe(CN)_6$ as exogenous electron acceptor. In Fig. 3, the initial amplitudes of absorption changes induced by each flash are depicted as a function of the wavelength in the range 250–370 nm. The difference spectrum exhibits pronounced peaks at 265 nm (negative) and 320 nm (positive) which are characteristic of the reductive formation of the X320-plastoquinone anion radical from plastoquinone [21,22]. Furthermore, the extent of the absorption changes induced by the first flash exceeds those due to subsequent flashes, which are almost independent of the number of flashes in the train. A clearly distinguished pattern is observed for the difference spectrum of the amplitudes measured 4.5 ms after each flash, as shown in Fig. 4. A similar situation to that depicted in Fig. 3 arises for the amplitudes due to the first flash which are significantly larger than those of the following flashes. However, in contrast to the initial amplitudes, the 4.5 ms amplitudes of the absorption changes caused by the third flash are markedly smaller than those of the second and fourth flash, which also slightly differ

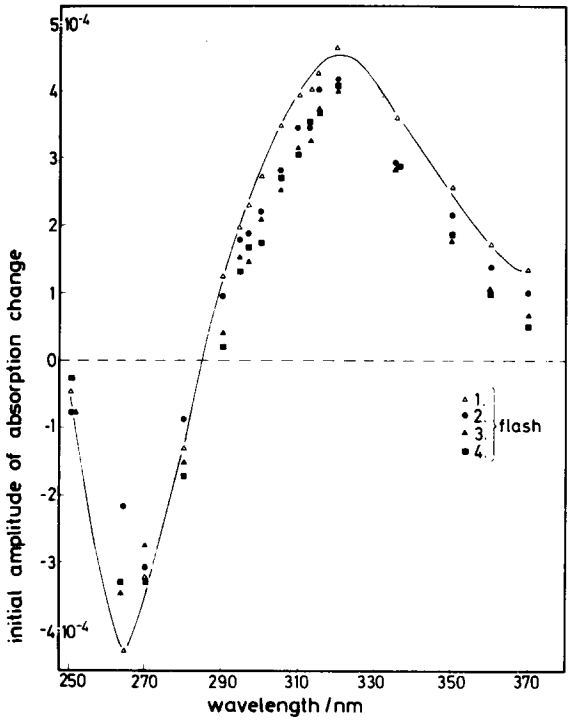

Fig. 3. Initial amplitudes of absorption changes, $\Delta A_n(0)$, induced by the first four flashes of a train in dark-adapted trypsinized chloroplasts as a function of wavelength. Electron acceptor, 0.5 mM $K_3Fe(CN)_6$; trypsin, 15 μg/ml chloroplast suspension; 32 samples were averaged.

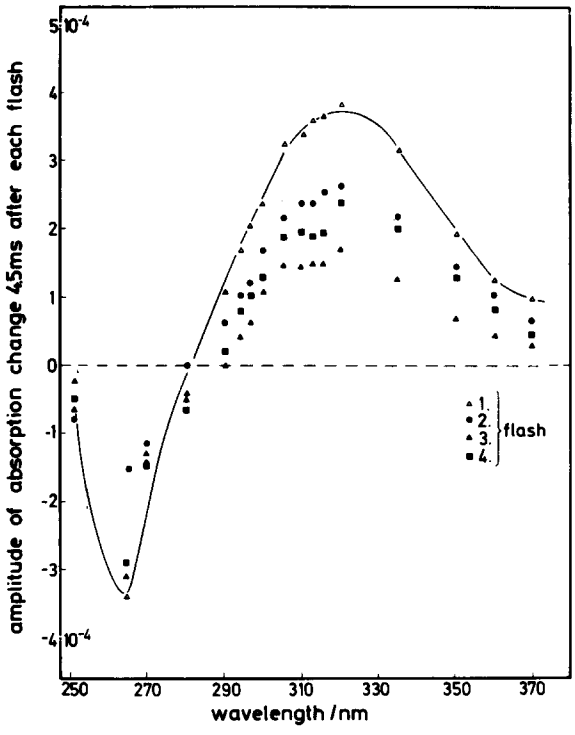

Fig. 4. Amplitudes at 4.5 ms after each flash, ΔA_n (4.5 ms), induced by the first four flashes of a train in dark-adapted trypsinized chloroplasts as a function of wavelength. Experimental conditions as in Fig. 4.

in their extent. If one admits that in trypsinized chloroplasts with 500 μM $K_3Fe(CN)_6$ as acceptor the turnover of X320 is independent of the number of flashes and that the reoxidation kinetics are slow [13] compared to the decay of the component Y-320, acting as a precursor of molecular oxygen, then the difference of the amplitudes $\Delta A_n^{4.5} = \Delta A_n(0) - \Delta A_n$ (4.5 ms) should reflect to a significant extent the difference spectrum between Y-320 and O_2. Furthermore, $\Delta A_n^{4.5}$ as a function of the flash number of the train is expected to oscillate in phase with the oxygen yield. The difference spectra of $\Delta A_n^{4.5}$ depicted in fig. 5 indicate a characteristic oscillation pattern. As expected the amplitudes are at a maximum at 320 nm. This might lead to the suggestion that a plastoquinone is involved in Y-320, but an unambiguous conclusion cannot be drawn on the basis of the present results (see Discussion)

The difference spectra reported in Fig. 5 indicate that already after the first flash a millisecond relaxation takes place which cannot be ascribed to Y-320, because after the first flash no oxygen evolution takes place. Besides an unresolved peak around 350 nm and the ratio of the positive and negative peak (0.47) being smaller than that of the $PQ^{\cdot-}/PQ$ couple [22], the difference spectrum of the millisecond relaxation after the first flash resembles that of $X320^{\cdot-}$. Therefore, this relaxation probably reflects a partial reoxidation of $X320^{\cdot-}$ by $K_3Fe(CN)_6$ which takes place in the millisecond range depending on $K_3Fe(CN)_6$ concentration [13]. Then the difference spectrum for component Y-320 vs. oxygen should be obtainable as the difference, $\Delta A_n^{4.5} - \Delta A_1^{4.5}$, provided that the contributions to $\Delta A_n^{4.5}$ which are not related to Y-320 remain nearly constant in the flash train. As the extent of Y-320 is expected to be maximal after the third flash, the difference $\Delta A_{3,1}^{4.5} = \Delta A_3^{4.5} - \Delta A_1^{4.5}$ is illustrated in Fig. 6 as a function of wave-

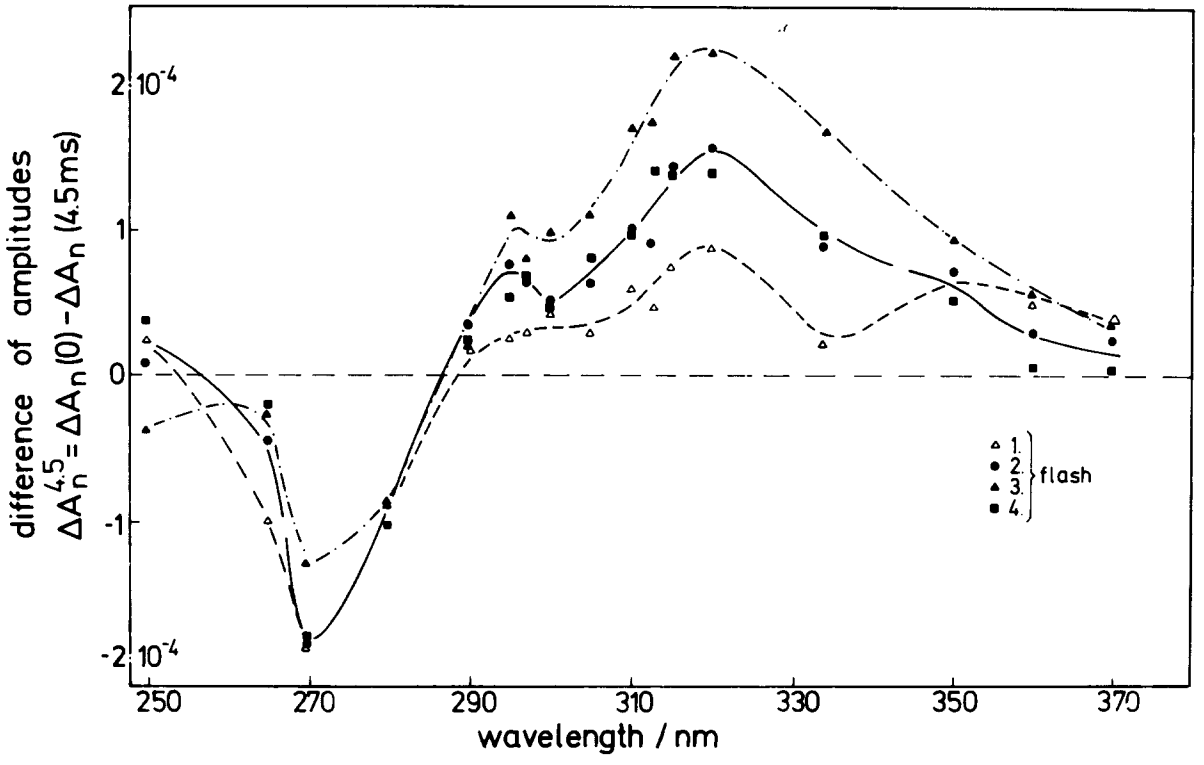

Fig. 5. Difference of initial amplitude and that 4.5 ms after each flash of absorption changes, $\Delta A_n^{4.5}$, induced by the first four flashes of a train in dark-adapted trypsinized chloroplasts as a function of wavelength. Experimental data from Figs. 4 and 5.

length. The data obtained exhibit a rather broad band, peaking around 320 nm. A negative band above 260 nm is not observed.

The small positive satellite band peaking around 265 nm might not necessarily reflect the turnover

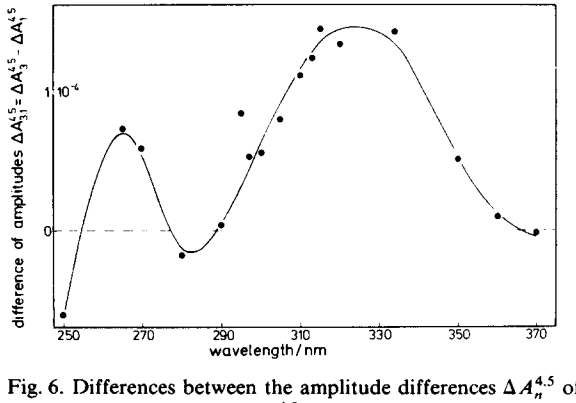

Fig. 6. Differences between the amplitude differences $\Delta A_n^{4.5}$ of the third and first flash, $\Delta A_{3,1}^{4.5}$, as a function of wavelength. Experimental data from Figs. 4 and 5.

of Y-320, because it is not observed in the second and fourth flash, i.e., for $\Delta A_{2,1}^{4.5}$ and $\Delta A_{4,1}^{4.5}$ (cf. Fig. 5). The origin of this band remains to be clarified.

In order to show that the interpretation of the difference, $\Delta A_{3,1}^{4.5}$ (Fig. 6), as indication of a precursor state Y-320 appears to be reasonable, difference spectra have been measured in trypsinized chloroplasts substituting $K_3Fe(CN)_6$ by benzyl viologen. In this case the reoxidation of $X320^-$ by external acceptors is eliminated so that only the slow reaction between S_2 and $X320^-$ leads to regeneration of X320 and no oxygen evolution takes place. In Fig. 7, the difference spectra $\Delta A(0)$ (top) and $\Delta A(4.5$ ms) (middle) are depicted. The resulting spectra of the difference, $\Delta A_n^{4.5}$, are shown at the bottom of Fig. 7. Rather small differences are observed, but even more important, the shape of the difference spectrum does not resemble those of Fig. 5 and no oscillations in the flash train of the extent are obtained. In contrast to the dif-

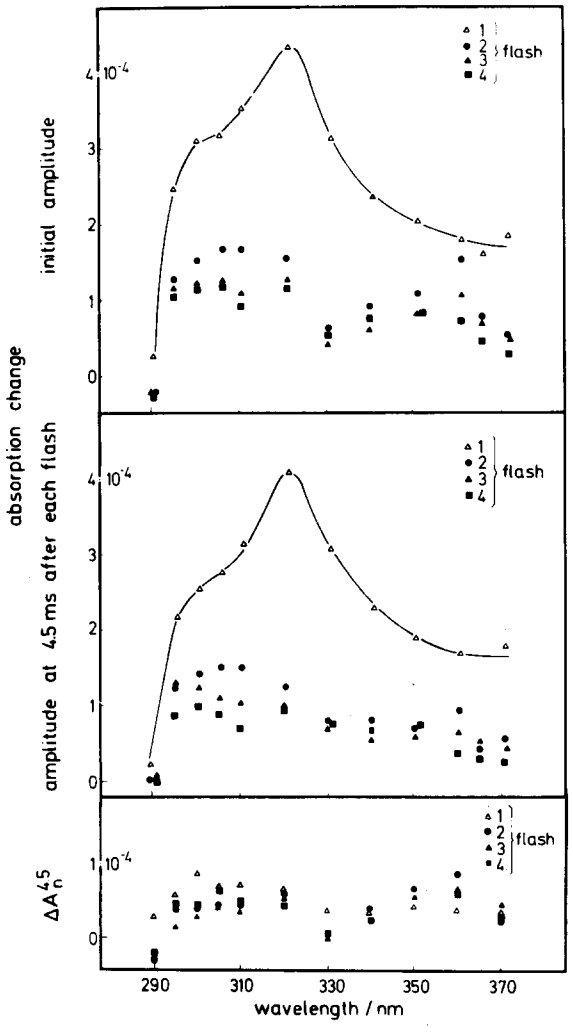

Fig. 7. Amplitudes of absorption changes induced by a train of four flashes in dark-adapted trypsinized chloroplasts as a function of wavelength. Electron acceptor, 0.1 mM benzyl viologen; trypsin; 15 μg/ml chloroplast suspension; 32 samples were averaged. Top, initial amplitude; middle, amplitude 4.5 ms after each flash; bottom, difference of initial amplitude and that 4.5 ms after each flash.

ference spectrum given in Fig. 6, $\Delta A_{3,1}^{4.5}$ appears to be even negative around 320 nm. As in the case of benzyl viologen, oxygen evolution is prevented due to the blockage of $X320^-$ reoxidation, and contributions of Y-320 to the difference spectrum are expected to be absent. Therefore, the failure to observe difference spectra with oscillating amplitudes like those of Fig. 5 supports the idea that the difference spectrum of Fig. 6 really reflects the spectral properties of Y-320, which is the precursor of photosynthetically evolved oxygen.

In normal chloroplasts in the presence of benzyl viologen absorption changes are observed (see Fig. 8) with difference spectra resembling those of Ref. 9. This spectrum significantly differs from that in trypsinized chloroplasts in the presence of $K_3Fe(CN)_6$, because the binary oscillations caused by the acceptor side are eliminated in the latter case. The turnover of water-splitting enzyme system Y involves not only the transient formation of Y-320 but also that of the intermediary redox states S_0, S_1, S_2 and S_3. The extent of those states oscillates in dark-adapted chloroplasts excited by a train of short flashes. Accordingly, the question arises as to whether these states are also characterized by absorption changes in the ultraviolet range. If one admits that in trypsinized chloroplasts in the presence of $K_3Fe(CN)_6$ the extent of the turnover of the primary plastoquinone acceptor X-320 giving rise to transient absorption changes in the ultraviolet region is always the same in each flash of the train, the initial amplitude of the absorption changes should oscillate. The oscillation pattern of the initial amplitudes expected under these circumstances would be dependent on the interpretation of Y-320. As these details are irrelevant to the general conclusion, they will not be discussed here. It remains to be mentioned that the data of Fig. 3 do not reveal a marked oscillation pattern. Accordingly, one has to conclude that also other S_i-state transitions are characterized by absorption changes in the ultraviolet. This interpretation is in line with previous conclusions [9–11]. As will be shown in a forthcoming paper, on the basis of the model of Kok et al. [3], it is generally possible to resolve by mathematical analysis the difference spectrum given in Fig. 4 into the spectra of the S_i states. However, as the procedure requires as basic set of data the difference of differences of absorption changes, the experimental errors are significant.

Discussion

The data presented in this paper reveal absorption changes in the ultraviolet, which are inferred to reflect the formation of the precursor state in the water-splitting enzyme system Y of photosyn-

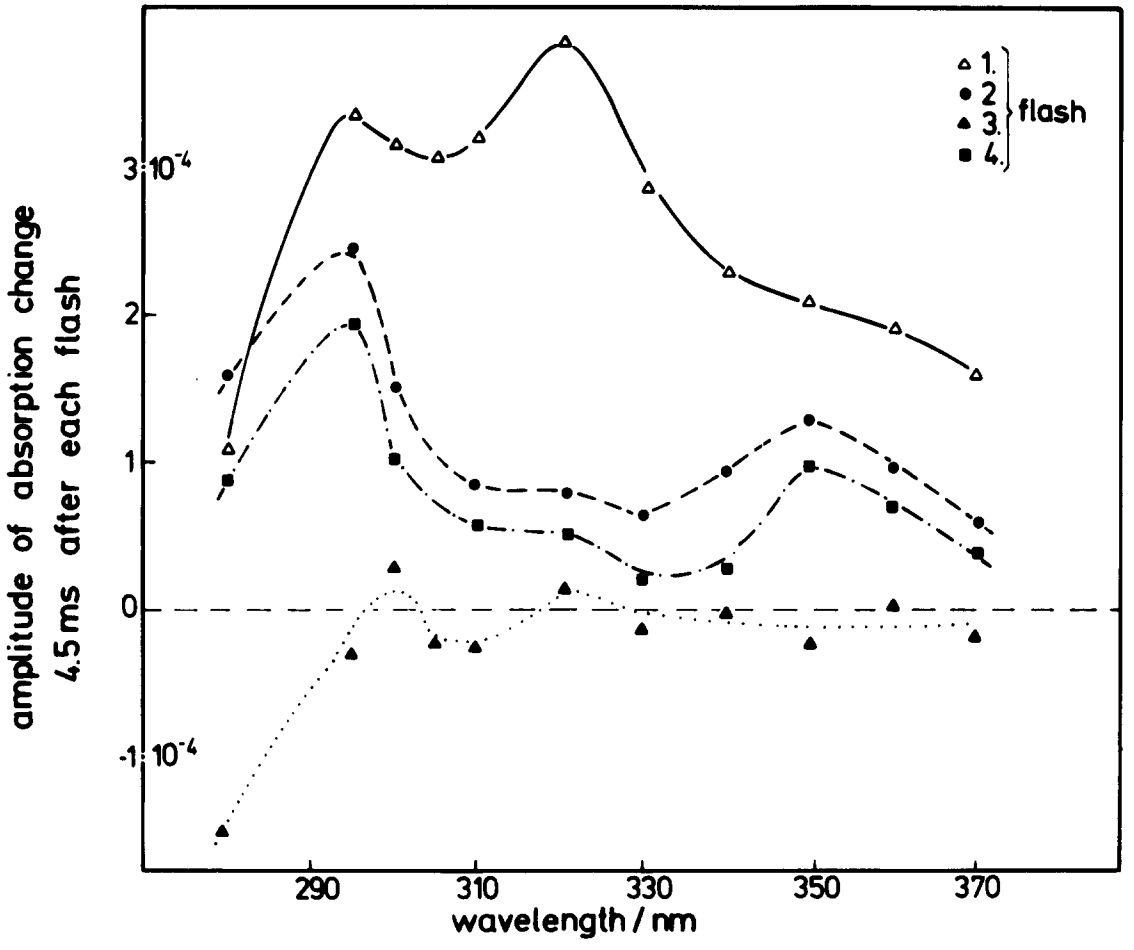

Fig. 8. Amplitudes at 4.5 ms after each flash, ΔA_n (4.5 ms), induced by the first four flashes of a train in dark-adapted normal chloroplasts. Electron acceptor, 0.1 mM benzyl viologen.

thetically evolved oxygen. This interpretation is based upon two experimental findings: (a) The selective disconnection of the reaction center of Photosystem II from the secondary plastoquinone acceptor B by mild trypsinization. In this way the binary oscillation pattern of absorption in the ultraviolet is eliminated, which is due to the coupling of one-electron photoprocesses at the reaction centers with two-electron reactions involving the plastoquinone/plastohydroquinone redox couple. (b) The differences of the transition kinetics of the reactions $S_i \overset{\oplus}{\rightarrow} S_{i+1}$, where $\oplus$ represents an oxidizing redox equivalent generated at Chl a_{II}^{+} within the reaction center. According to EPR data reported by Babcock et al. [25], the reactions $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ take place with half-lifetimes of less than approx. 100 μs, whereas the $S_3 \rightarrow S_4$ transition is limited by kinetics with a half-time of 1 ms. The latter value corresponds to the rate of oxygen release from system Y [24]. Accordingly, the oxygen formation appears to be limited kinetically by the oxidation of S_3, whereas the formation of S_3 from S_2 takes place with a half-life of 400 μs. The time resolution of our equipment was of the order of 100 μs, so that in a reasonable approximation the apparent initial amplitude involves absorption changes due to the transitions $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$. On the other hand, the relaxation kinetics, which oscillate in extent synchronously

with the oxygen yield in a flash train, are characterized by a half-time of 1 ms. As the reaction pattern of the donor side of system Y was found to be almost invariant with respect to mild trypsinization [18,23], it appears rather logical to identify these absorption changes as the precursor state of photosynthetically evolved oxygen, tentatively referred to as Y-320. The correspondence between the half-lifetimes of S_3 oxidation [25] and the overall rate of oxygen release [24] further supports the idea that Y-320 might be substantiated as S_3 (vide infra). A possible interference of the kinetics of the $S_2 \rightarrow S_3$ transition ($t_{1/2} = 400$ μs) can be excluded because the extent of this reaction should be at a maximum in the second flash. However, neither the rise nor the decay kinetics of the absorption change induced by the second flash exhibit a significant 400 μs component (see Fig. 1). Therefore, the oxidation of S_2 to S_3 is inferred to cause only very small absorption changes in the range 260–370 nm.

The interpretation that the difference spectrum of Fig. 6 reflects the precursor state Y-320 of photosynthetically evolved oxygen is corroborated by the finding that DCMU does not seriously modify the oscillation pattern in trypsinized chloroplasts (data not shown), whereas substitution of $K_3Fe(CN)_6$ by benzyl viologen or addition of the powerful ADRY agent ANT-2p, acting in the same manner as ANT-2s, completely suppresses the oscillation (see Ref. 14). The basic conclusion that the precursor state Y-320 is characterized by an absorption in the ultraviolet range is in line with previous studies indicating the turnover of the watersplitting enzyme system Y to give rise to absorption changes in this spectral range [9–11]. Velthuys [11] interpreted the difference of absorption changes observed in dark-adapted chloroplasts in the absence and presence of hydroxylamine to reflect the oxidation of a component L during the $S_1 \rightarrow S_2$ transition and the reduction in the course of the reaction $S_4 \rightarrow S_0$. His finding that the extent of relaxation kinetics with a half-time of 1 ms is at a maximum after the third flash corresponds with our findings (see Fig. 1). However, the differences observed in the second and fourth flash do not quantitatively fit our data. Furthermore, the difference spectrum presented in Fig. 6 differs from that of the L oxidation (see Fig. 3 of Ref. 11). These discrepancies might be caused by the differences in procedures applied for elimination of the binary oscillations of the acceptor side of Photosystem II. The NH_2OH method (control minus NH_2OH-treated chloroplasts) does not necessarily provide an unambiguous elimination of all absorption changes which are not related to the turnover of system Y, because even the mechanism of hydroxylamine treatment appears to be not completely resolved. Van Best and Duysens [26] conclude that it blocks the electron transport between D_1 and Chl a_{II} so that Chl a_{II}^+ becomes reduced via another endogenous electron donor. On the other hand, Etienne et al. [27] found that NH_2OH treatment gives rise to EPR signal IIf which is identical to that observed in Tris-washed chloroplasts, thus indicating that the electron transfer from D_1 to Chl a_{II}^+ remains only retarded compared to normal chloroplasts. If the reactivity of D_1 is significantly modified by NH_2OH, the difference method should also give rise to absorption changes due to the turnover of D_1 provided that D_1 and D_1^+ differ in their ultraviolet absorption spectra. Therefore, further experiments are required in order to prove that the observed differences (control minus NH_2OH-treated chloroplasts) of the absorption changes are exclusively attributable to redox changes within the water-splitting enzyme system Y.

If one accepts that the difference spectrum of Fig. 6 really does reflect the ultraviolet absorption of the precursor Y-320 vs. the S_0 state of system Y, the question arises as to whether this spectrum could provide any information about the chemical nature of the precursor Y-320. According to a molecular model proposed previously [4,6] for the four-step univalent redox mechanism of photosynthetic water oxidation, two types of reactions have to be considered for the interpretation of Y-320:

$$(H_2O_2)^{**}M^+ \xrightarrow[H^+]{k(\text{Chl } a_{II}^+)} (H^+ \cdots O^{\tau}{}_2)^{**}M^+ \underset{H^+}{\overset{k_b}{\rightleftharpoons}} (O_2)^{**} + M \qquad (1)$$

$$(O_2)^{**} + 2H_2O \xrightarrow{k_{O_2}} O_2 + 2(H_2O) \qquad (2)$$

In this model S_3 is postulated to be a complexed hydrogen peroxide (referred to as cryptoperoxide)

coupled to another redox group staying in the oxidized state M^+. After generation of Chl a_{II}^+ the cryptohydrogen peroxide becomes oxidized via a two-electron abstraction involving electron transfer to M^+ from intermediary formed cryptosuperoxide (Eqn. 1). Subsequently, complexed oxygen is assumed to be released via an exergonic ligand-exchange reaction (Eqn. 2). Therefore, depending on the ratio of the rate constants k(Chl a_{II}^+) characterizing the oxidation of S_3 by Chl a_{II}^+, k_b describing the formation of bound oxygen and k_{O_2} for the ligand-exchange reaction, state Y-320 represents either the 'species' $(H_2O_2)^{**}M^+$ or $(H^+ \cdots O_2^-)^{**}M^+$ or $(O_2)^{**} + M^+$ vs. $(H_2O)^* + M$. If one takes into account the kinetic data of EPR measurements presented by Babcock et al. [25] as well as the release of oxygen described by Joliot et al. [24], then the relation should be valid: $k(\text{Chl } a_{II}^+) \ll k_b, k_{O_2}$. Therefore, the spectrum of Fig. 6 should reflect the absorption difference between the states $(H_2O_2)^{**}M^+$ and $(H_2O)^*M$.

With respect to the mechanism of the water-splitting enzyme system Y, the assumption $k(\text{Chl } a_{II}^+) \ll k_b$ appears to be reasonable if one compares the sequence of water oxidation with that of the reverse reaction catalyzed in cytochrome oxidase. Very recently, it was found that the primary step in the reduction sequence leading from O_2 to H_2O in cytochrome oxidase is the formation of a loosely bound complex between O_2 and the Fe^{2+} of cytochrome a_3, which closely resembles oxyhemoglobin [28]. It therefore appears to be an attractive speculation to assume that the final stage of oxygen evolution also involves a loosely bound O_2. Oxygen binding could be either mononuclear as in cytochrome oxidase or hemoglobin or binuclear as in hemocyanins. In the latter case, O_2 was found to be complexed as a peroxide by two copper centers of the enzyme, which is characterized by a peak around 340 nm [20]. In cytochrome oxidase, O_2 becomes reduced to the peroxide level by an almost simultaneous electron transfer from Fe^{2+} of cytochrome a_3 and from $Cu_{a_3}^+$. A reverse reaction sequence could be realized in the water-splitting enzyme system Y. In this case, the peroxide state was postulated to be complexed binuclearly at two manganese centers (it should be emphasized that the binuclear complexation is the crucial point, not the nature of the metal centers) in contact with the oxidized group M^+ [4,6]. The redox transition from the peroxide level to molecular oxygen could occur via a quasi-concerted two-electron transfer similar to that of cytochrome oxidase, but in the reverse direction. The oxidative process in system Y would be triggered by electron abstraction from a manganese center through photooxidized Chl a_{II}^+ and the subsequent second electron transfer to M^+. This is the basic idea of the molecular model described previously [4,6]. Therefore, the assumption of a 'concerted' two-electron transfer favors the idea that $k_b \gg k(\text{Chl } a_{II}^+)$.

Concerning the interpretation of the difference spectrum of Fig. 6, the positive peak around 320 nm might suggest participation of plastoquinones in the reaction sequence of system Y, which was inferred to occur based on the finding that plastoquinone is required for restoration of the oxygen-evolving capacity in plastoquinone-extracted thylakoids [7,8]. It was proposed that PQ forms a stable Mn(III)-PQ^- complex and that molecular oxygen formation and release occur via the cleavage of four such groups containing additionally OH^-, i.e., $[\text{Mn(III)-PQ}^-]_4(OH^-)_2 \rightarrow 4\text{Mn(II)} + 2PQ + 2PQ^{2-} + O_2 + 2H^+$ (see Ref. 8). Analogously, the plastohydroquinone/plastosemiquinone system could be the M/M^+ couple, because the redox potential of PQH_2/PQH is known to be strongly dependent on the environment, especially on the local pH [30]. A substantiation of the M/M^+ couple with a special plastohydroquinone/plastosemiquinone system would involve the assumption of a rather high stability of the plastosemiquinone species in order to account for the exceptionally long lifetime of state S_1 in system Y. Plastoquinone molecules complexed in a certain manner exhibit very special properties, as is well known for the primary plastoquinone acceptor of Photosystem II, which acts as a one-electron carrier system only [31]. Furthermore, the secondary plastoquinone B in its semiquinone form was found to be rather stable [27]. The spectrum of Fig. 6 is not in a contradiction with the substantiation of M/M^+ as plastohydroquinone/plastosemiquinone, because the latter system does not contain a pronounced negative peak around 265 nm, in marked contrast to the system plastoquinone/plastosemiquinone. However, the spec-

trum of Fig. 6 does not prove this idea, because other components which might be likely candidates to be the M/M^+ couple also undergo absorption changes in the ultraviolet.

If the spectrum of Fig. 6 exclusively indicates the M/M^+ redox couple, the initial amplitudes should oscillate. A small oscillation has been observed (maximum at first and fifth flash, data not shown), but a quantitative agreement with a theoretical pattern could not be achieved. Therefore, further investigation is necessary in order to unravel the chemical nature of the precursor state Y-320.

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