Indirect Evidence for a Very Fast Recovery Kinetics of Chlorophyll-a_{II} in Spinach Chloroplasts

M. Gläser, Ch. Wolff †, and G. Renger

Max-Volmer-Institut für Physikalische Chemie und Molekularbiologie
der Technischen Universität Berlin

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The 690 nm absorption change reflecting the turnover of the system-II-reaction center chlorophyll, Chl-a_{II} (often referred to as P 680), has been investigated under different experimental conditions in spinach chloroplasts. A comparison was made with oxygen evolution and with absorption changes of Chl-a_I measured at 703 nm, both indicating the number of electrons produced by system II. It was found:

1. The dependency on actinic flash intensity of the initial amplitudes of the measured 690 nm absorption change, ΔA_0(Chl-a_{II}), markedly differs for normal and for Tris-washed chloroplasts, respectively.
2. The saturation curve of ΔA_0(Chl-a_{II}) in Tris-washed chloroplasts is similar to that for the total amplitude of the 703 nm absorption change, ΔA_0(Chl-a_I), in normal chloroplasts, and can be described by an exponential function. On the other hand, ΔA_0(Chl-a_{II}) in normal chloroplasts exhibits a more complex biphasic dependency and much higher flash intensities are required for saturation.
3. Under repetitive flash group excitation and in the presence of an ADRY (= acceleration of the deactivation reactions of the water-splitting enzyme system Y)-reagent the initial amplitude of the 690 nm absorption change oscillates in the same characteristic pattern as the oxygen evolution.
4. The initial amplitude of the 690 nm absorption change, ΔA_0(Chl-a_{II}), in Tris-washed chloroplasts becomes significantly smaller (more than 50%) by the addition of system-II-electron donors (benzidine, *p*-phenylendiamine, tetraphenylboron), whereas the total amplitude of the 703 nm absorption change, ΔA_0(Chl-a_I) increases 3–4-fold.

In order to explain these results, the existance of a very fast reduction kinetics of Chl-a_{II}^+ is postulated, which is not detectable by our measuring equipment. The half time of this reaction is ≤ 1 μs. Reaction centers with the very fast "undetected" Chl-a_{II}^+-reduction are photochemically transformed into slower one by double hit processes with a comparatively low quantum yield. Furthermore, it is inferred, that the dark recovery kinetics of Chl-a_{II} is dependent on the charge accumulation state of the watersplitting enzyme system Y. This phenomenon is shown to explain also the oscillation pattern of delayed fluorescence. On the basis of the present results two alternative reaction schemes for the functional organization of the electron transport on the donor side of system II are discussed.

Introduction

The reaction center of system II in its active state, symbolized by C_{II}*, generates *via* photochemical processes holes of an oxidizing power strong enough for water cleavage. Since 1967 absorption changes in the range of 680 – 690 nm have been discovered by Witt and coworkers [1–4] which were inferred to reflect the reversible photobleaching of a special chlorophyll-a belonging to the reaction center of system II. Therefore, this component was designated as chlorophyll-a_{II} (Chl-a_{II}). It was found that in short flashes Chl-a_{II} is very rapidly bleached and subsequently recovers in the dark with a half life time of approx. 200 μs. Recent experiments showed the existence of a second faster component of the regeneration kinetics of Chl-a_{II} with a half life time of 35 μs [3]. Furthermore, it was inferred that electronically excited Chl-a_{II} acts within the reaction center of system II as the primary electron donor [4, 5]. Thus, the bleaching has been ascribed to a photooxidation of Chl-a_{II}, whereas the recovery was assumed to reflect the

Requests for reprints should be sent to Dr. G. Renger, Max-Volmer-Institut für Physikalische Chemie und Molekularbiologie der Technischen Universität Berlin, Straße des 17. Juni 135, *D-1000 Berlin 12*.

† Deceased on July 22th, 1975.

* The symbol C_{II} includes the functional (primary electron donor and acceptor) and structural elements of the molecular arrangement responsible for the photochemical charge separation: $C_{II} \xrightarrow{\langle\varepsilon\rangle} C_{II}^{\pm}$, where $C_{II}^{\pm}$ characterizes the charged (photochemically inactive) state ("–" symbolizes the reduced primary acceptor and "+" the oxidized primary donor) of system-II-reaction center and $\langle\varepsilon\rangle$ an exciton generated by light quantum absorption.

kinetics of the reduction of Chl-a_{II}^+ by the intermediary electron carriers located between the reaction center and the enzyme system responsible for the wateroxidation [4]. The destruction of the water-splitting enzyme system Y by various treatments did not inhibit the functional integrity of C_{II} itself even under repetitive excitation conditions [6, 7]. Hence, it was concluded that either a fast cyclic electron flow around system II or an internal back reaction of the order of $100-200\ \mu s$ is responsible for the fast photochemical turnover rate of the reaction centers of system II [7]. The internal back reaction at the centers in the state C_{II}^{+} has been postulated to be the unique mechanism for the generation of excitons giving rise to delayed fluorescence [8]. It was found, that Tris-washed chloroplasts exhibit a pronounced delayed fluorescence emission in the range of $100-200\ \mu s$ [9], which supports evidence for the occurance of an internal back reaction at C_{II}^{+}. If one supposes that Tris-treatment does not seriously modify the reactivity of the reaction centers, then the kinetics of the back reaction in normal chloroplasts should be also of the order of $200\ \mu s$. However, as the back reaction leads to the dissipation of electrons and holes, respectively, a $100-200\ \mu s$ half time for this process and a competing $35\ \mu s$ kinetics for the non-dissipative removal of the hole from Chl-a_{II}^+ are inconsistent with a quantum yield very close to unity for system II electron transport [10]. Furthermore, Duysens and coworkers [11, 12] discovered very rapid components of prompt and delayed fluorescence, too fast to be explainable by a $35\ \mu s$ kinetics of Chl-a_{II}^+ reduction. In order to resolve these discrepancies the recovery kinetics of Chl-a_{II} was reinvestigated by the application of different experimental conditions. The obtained results presented here provide indirect evidence for the existence of a rapid electron transfer from a one-electron donor with a halflife time of $\leqq 1\ \mu s$. This electron donor (D_1) functionally connects Chl-a_{II} with the water-splitting enzyme system Y.

The existence of a very rapid rereduction in the dark of Chl-a_{II}^+ explains the high quantum yield of photosystem-II-electron transport as well as the fast components of prompt and delayed fluorescence, respectively.

Materials and Methods

Normal and Tris-washed chloroplasts were prepared from market spinach on the basis of the methods of Winget *et al.* [13] and Yamashita and Butler [14], respectively, as is described in ref. 15.

The reaction mixture for the measurements of the absorption changes contained: chloroplasts ($5\ \mu M$ chlorophyll), $100\ \mu M$ benzylviologen, 2 mM $MgCl_2$, 2 mM NH_4Cl and 20 mM N-tris-(hydroxymethyl)methylglycine (Tricine)-NaOH, pH = 7.2.

The reaction mixture for the oxygen measurements contained chloroplasts ($50\ \mu M$ chlorophyll) and $250\ \mu M$ $K_3[Fe(CN)_6]$ as electron acceptor instead of benzylviologen, other additions as for the optical measurements.

The absorption changes were recorded by a repetitive flash photometer similar to that described in ref. 1 and modified by the use of high frequency modulated detecting light. This method introduced by Buchwald [16, 17] eliminates fluorescence artefacts due to the actinic flashes. In some measurements this artefact was eliminated by a subtraction method as is reported in ref. 1. In a Fabri-Tek 1072 usually $2048-16384$ signals were averaged per measurement but the sample was changed after each 4096 flashes. The electrical bandwidth of the apparatus was 30 kHz.

Photosynthesis was excited by ultra short repetitive flashes [18] of a duration of 400 ns except for Fig. 2 and for the oxygen measurements, where the flash duration was $20\ \mu s$ and a frequency of 4 Hz, except for Fig. 3 where other excitation conditions were used (s. inside Fig. 3). The flash light passed a Schott filter BG 23/5. The optical pathlength of the cuvette was 20 mm, the intensity of the monitoring light 100 erg/cm² s, optical bandwidth $\Delta\lambda = 5$ nm, continuous back ground light (720 nm, $\Delta\lambda = 15$ nm) was applied with an intensity of about 20 000 erg/cm²·c.

Oxygen measurements were performed as described in ref. 19.

Results

The time course of the absorption changes at 690 nm in normal and in Tris-washed chloroplasts is shown in the left side of Fig. 1. In order to avoid in the μs-range any contribution of the chlorophyll-a_I (Chl-a_I) recovery kinetics chloroplasts were illuminated with far red background light as is described in ref. 3. For comparison on the right side the time course of the absorption change at 703 nm is depicted in a 200-fold larger time scale. It shows a rather slow pattern, with a zero-line which is shifted downwards due to the high degree of oxidation (approx. 65%) of Chl-a_I by far red preillumination [20].

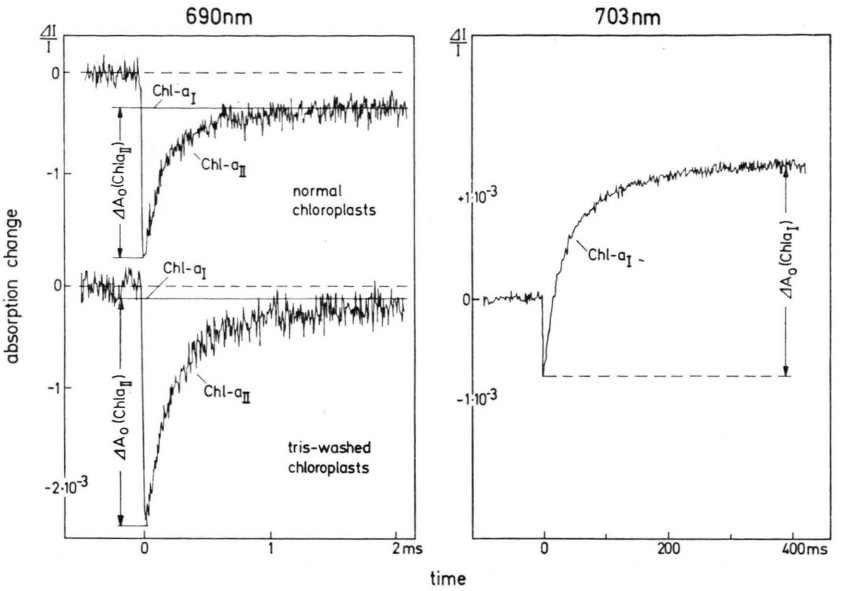

Fig. 1. Absorption changes at 690 nm and 703 nm as a function of time in spinach chloroplasts. At $t=0$ a short flash was fired. Experimental details as described in Materials and Methods.

The results indicate that the initial amplitude of the 690 nm absorption change, ΔA_0 (Chl-a_{II}), which is assumed to reflect the formation of Chl-a_{II}^+ [4], is significantly higher in Tris-washed chloroplasts compared to normal chloroplasts. On the other hand, the overall recovery kinetics of Chl-a_{II} in the range of $10-1000$ μs is not seriously modified by the Tris-treatment. The rough invariance of the detectable overall recovery kinetics suggests that the 690 nm absorption change reflects the same phenomenon in normal and in Tris-washed chloroplasts, respectively, namely the turnover of Chl-a_{II}.

As Tris-washing cannot be assumed to enhance the number of system II reaction centers, the increase of the initial amplitude ΔA_0(Chl-a_{II}) of the 690 nm absorption change (s. Fig. 1) must be explained by other effects. It could be possible that in normal, but not in Tris-washed chloroplasts, there exists an even faster recovery kinetics * of Chl-a_{II} which escaped our detection due to the limited time resolution of the measuring device. Similarly, normal and Tris-washed chloroplasts, respectively, could differ in the dependency on actinic flash intensity of the detected part of Chl-a_{II}^+ formation.

To clarify these points, first the initial amplitudes of Chl-a_{II}, ΔA_0(Chl-a_{II}) (s. Fig. 1), have been measured in normal and in Tris-washed chloroplasts as a function of the actinic flash intensity I_f. The obtained results are given in Fig. 2 in comparison with the total amplitude of Chl-a_I, ΔA_0(Chl-a_I) in normal chloroplasts (s. Fig. 1). Under the applied experimental conditions the total amplitude of the 703 nm absorption change, ΔA_0(Chl-a_I), was shown to indicate the number of electrons produced by system II [20]. Recent experiments (20 a) revealed small differences between the total numbers of Chl-a_I and Chl-a_{II}, respectively. However, this

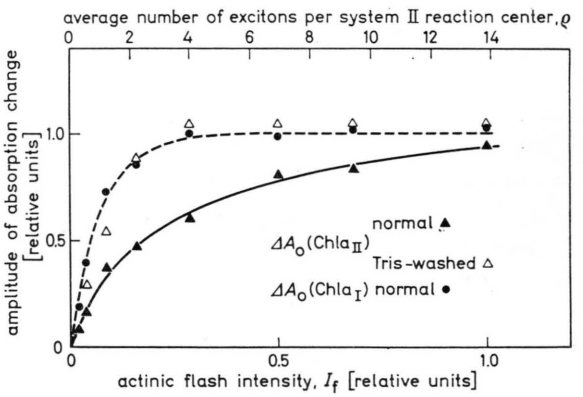

Fig. 2. Amplitudes of the absorption changes at 690 nm, ΔA_0(Chl-a_{II}), in normal and in Tris-washed chloroplasts, and at 703 nm, ΔA_0(Chl-a_I), in normal chloroplasts as a function of actinic flash intensity. The calibration of actinic flash intensity, I_f, into units of excitons per system II reaction center, ϱ (s. Eqn (1)), is given in the top abscissae (for details s. text). Experimental details as described in Materials and Methods.

* In the present paper this hypothetical component is referred to as "undetected" in contrast to the detected components ΔA_0(Chl-a_{II}) which can be measured by our apparatus.

fact will be neglected, because its effect is within the accuracy limit of our present data. That means, ΔA_0(Chl-a_I) reflects the total number of the photo-chemical turnovers at the system-II-reaction centers in normal chloroplasts, where the dissipation of photoproducts at system II by cycling reactions is negligibly small (s. ref. 10). Therefore, the close similarity of the saturation curves for ΔA_0(Chl-a_I) in normal chloroplasts and ΔA_0(Chl-a_{II}) in Tris-washed chloroplasts indicates that the photochemical quantum yield for system II is not changed by Tris-treatment, likewise the detected absorption change reflects the total Chl-a_{II}^+-formation. On the other hand the corresponding saturation curves for ΔA_0(Chl-a_I) and ΔA_0(Chl-a_{II}), respectively, significantly differ in normal chloroplasts. This discrepancy can be resolved by two assumptions: a. There exists in normal chloroplasts a rapid rereduction reaction of Chl-a_{II}^+ giving rise to a relaxation component of the 690 nm absorption change too fast to be detectable by our equipment. b. The contribution of this hypothetical rapid rereduction component to the total absorption change of Chl-a_{II} decreases with increasing intensities of the actinic flashes. This could be achieved by double hits transforming reaction centers of system II with an "undetected" fast Chl-a_{II} recovery into slower ones (35 μs and 200 μs kinetics).

For the sake of simplicity the following analysis is based on a separate unit model for excitation energy transfer in system II. Each photosynthetic unit of system II is assumed to contain only one trap, the reaction center, and no energy transfer occurs between the units. Furthermore, the photochemical quantum yield of the first hit is 1 (in close agreement with the experimental data, s. ref. 10) and the quantum yield of a following hit occuring only at centers with the fast "undetected" Chl-a_{II} recovery is Φ^*. Then, if the rapid undetected kinetics is fast in comparison to the flash duration, one obtains (s. Appendix) for the dependency on actinic flash intensity of the "detected" initial amplitude of Chl-a_{II}, ΔA_0(Chl-a_{II}):

$$\frac{\Delta A_0(\text{Chl-a}_{II})}{[\Delta A_0(\text{Chl-a}_{II})]_{max}} = 1 - a_1 \cdot e^{-\varrho} - \frac{a_2}{1-\Phi^*} \cdot (e^{-\Phi^* \cdot \varrho} - \Phi^* \cdot e^{-\varrho}), \quad (1)$$

where ϱ denotes the average number of excitons, produced by a flash of intensity I_f per photosynthetic unit of system II, which are able to reach a system-II-reaction center, irrespective of its functional state, a_1 and a_2 are the fractions of reaction centers with a slow and fast recovery, respectively, of Chl-a_{II} after single hit excitation. It must be emphasized, that Eqn (1) is exactly valid only if the absorption of the sample is negligibly small. This is not realized under our experimental conditions. However, a suitable calibration method is available.

As effects of multiple excitations in the ns- and subnanosecond range (s. ref. 21) can be neglected under our excitation conditions (flash duration 20 μs, intensity I_f low enough) ϱ is a linear function of the actinic flash intensity. In order to transform the actinic flash intensity into units of ϱ the saturation curve for the total amplitude of the 703 nm absorption change, ΔA_0(Chl-a_I), has been used. Taking into account the above mentioned assumptions (seperate units, quantum yield for the photochemical charge separation = 1) theoretically a simple exponential curve

$$\Delta A_0(\text{Chl-a}_I)/[\Delta A_0(\text{Chl-a}_I)]_{max} = 1 - e^{-\varrho}$$

results. Experimentally also a simple exponential relationship between ΔA_0(Chl-a_I)/$[\Delta A_0$(Chl-a_I)$]_{max}$ and I_f, the actinic flash intensity, was found, so that the calibration can be easily performed. As the same excitation conditions are applied also for the measurements at 690 nm, the same calibration method holds. On the basis of this procedure the experimental data of ΔA_0(Chl-a_{II}) as a function of actinic flash intensity for normal chloroplasts can be described by Eqn (1). The best fit is obtained for $a_1 = 0.4$, $a_2 = 0.6$ and $\Phi^* = 0.17$. That means: a. After single hit excitation about 60% of all system II reaction centers C_{II} are characterized by a fast rereduction of photoinduced Chl-a_{II}^+ which is not detectable by our measuring device, whereas only 40% recover *via* the slower kinetics of 35 μs and 200 μs. b. At higher exciton fluxes the transformation of reaction centers of system II with a fast Chl-a_{II} recovery kinetics into slower ones takes place by a second excitation with a quantum yield of $\Phi^* = 0.17$.

The fast reduction of Chl-a_{II}^+ requires the rapid electron transfer from a natural electron donor (referred to as D_1) located in the pathway between Chl-a_{II} and the watersplitting enzyme system Y (s. Discussion, Fig. 5). The half time of this electron transfer is estimated to be $\leqq 1$ μs. As this kinetics is very rapid in comparison to the 35 μs and 200 μs

reactions, the slower components can only appear when the fast ($\leqq 1\,\mu s$) reaction between Chl-a_{II}^+ and the donor D_1 is blocked. This would be caused either by changes which modify the functional connection Chl-a_{II}-D_1 or by oxidation of D_1. In the latter case it would be reasonable to assume that D_1 equilibrates with the oxidation states of the water-splitting enzyme system Y (S-states, s. ref. 22), *i. e.* in the states S_2 and S_3 the redox level of D_1 is shifted towards the oxidized form. Therefore, as the "undetected" fast ($\leqq 1\,\mu s$) component only occurs if D_1 is in the reduced state, one would expect, that the amplitudes of the slower components (35 μs + 200 μs phase) oscillate in a similar way as the S-states of the water-splitting enzyme system Y.

This oscillation pattern can be observed only in dark adapted chloroplasts by excitation with a train of short saturating flashes. In order to be able to measure the anticipated effect under repetitive flash excitation conditions an ADRY-agent (s. ref. 19) was applied which is known to accelerate the dark relaxation of S_2 and S_3 up to two orders of magnitude in spinach chloroplasts [23, 24].

Fig. 3 shows the dependency on the flash number in a train of the average oxygen yield per flash and of the initial amplitude of the detected 690 nm absorption change, ΔA_0(Chl-a_{II}), respectively, in the presence of 2-(3-trifluoro-4-chloro) anilino-3,5-dinitrothiophene (ANT 2p). It is seen that both, the average oxygen yield per flash and the initial amplitude of the detected Chl-a_{II}-absorption change oscillate synchronously. This confirms the above mentioned assumption, that the Chl-a_{II} recovery kinetics depend on the charge accumulation state (S-states) of the water-splitting enzyme system Y.

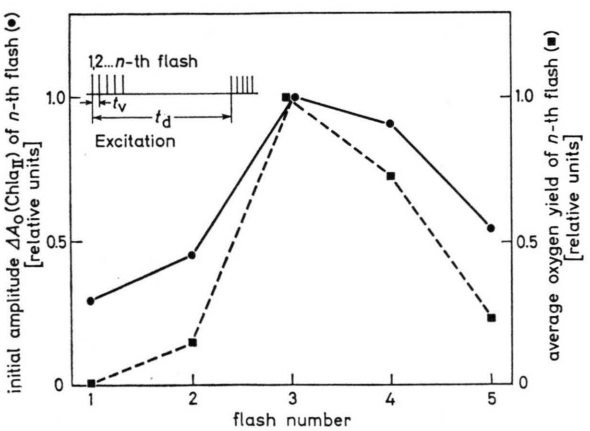

Fig. 3. Initial amplitudes of the measured ("detected") absorption change at 690 nm, ΔA_0(Chl-a_{II}), and average oxygen yield per flash as a function of the flash number of a train in spinach chloroplasts in the presence of 2-(3-chloro-4-trifluoromethyl) anilino-3,5-dinitrothiophene (ANT 2p) (0.03 μM and 0.5 μM, respectively). The data are normalized for the corresponding values of the 3rd flash in the train. Excitation conditions as indicated in the figure. Other details as described in Materials and Methods.

Hence, the recovery kinetics of Chl-a_{II} seems to be controlled by the redox state of D_1 rather than by the functional states of the Chl-$a_{II} - D_1$-connection.

If the electron transport from water to the reaction center C_{II} is interrupted by Tris-treatment, then the intermediary redox carriers including D_1 should attain a high population of their oxidized states by repetitive excitation with short flashes. Therefore, the fast ($\leqq 1\,\mu s$) "undetected" recovery kinetics of Chl-a_{II} would be absent. This effect is really observed, as is shown by the close correspondence of the saturation curves for ΔA_0(Chl-a_{II}) in Tris-washed and ΔA_0(Chl-a_I) in normal chloroplasts, respectively. On the other hand, one could anticipate that the addition of artificial system II electron donors to Tris-washed chloroplasts shifts the redox levels of the intermediary electron carriers, especially of D_1, towards their reducing side, thereby restoring the fast ($\leqq 1\,\mu s$) "undetected" recovery kinetics of Chl-a_{II}. If this assumption is correct, then in Tris-washed chloroplasts addition of a system II electron donor would lead to a decrease of the initial amplitude ΔA_0(Chl-a_{II}), because of the restoration of the fast Chl-a_{II}^+ rereduction kinetics. Simultaneously, the total amplitude at 703 nm, ΔA_0(Chl-a_I), has to increase due to the regeneration

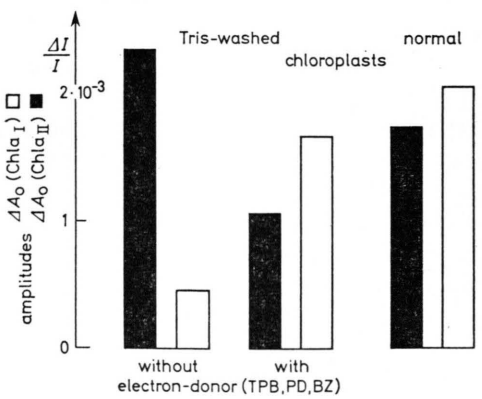

Fig. 4. Amplitudes of the absorption changes at 690 nm, ΔA_0(Chl-a_I), in Tris-washed chloroplasts in the absence and presence of artificial system II electron donors, respectively, and in normal chloroplasts. Experimental conditions as described in Material and Methods.

of the linear electron transport by these donors as has been already reported in ref. 15. The experimental data presented in Fig. 4 confirm this to be really the case. For comparison on the right side the amplitudes ΔA_0(Chl-a_{II}) and ΔA_0(Chl-a_I) are given for normal chloroplasts.

These results provide further evidence for the dependency of the initial amplitude of the "detected" Chl-a_{II} absorption change, ΔA_0(Chl-a_{II}), on the redox state of the donor side of system II.

Discussion

From the present results two conclusions can be drawn:

1. There exists indirect evidence for a very fast component of the dark reduction of photooxidized Chl-a_{II}^+ by a natural electron donor D_1 which is functionally connected with the water splitting enzyme system Y. The half life time of this reaction is of the order of $\leqq 1$ μs.

2. The reduction kinetics of Chl-a_{II}^+ are complex and are dependent on the functional state of the watersplitting enzyme system Y. The postulation of a very fast Chl-a_{II}^+-reduction of the order of $\leqq 1$ μs is in close correspondence with similar conclusions drawn on the basis of measurements of prompt and delayed fluorescence, respectively [11, 12]. Therefore, the existence of the fast Chl-a_{II} recovery after photooxidation is made highly probable by different lines of indirect evidence. Furthermore, it is known, that the electron transfer from D_1 to Chl-a_{II}^+ is intimately related to the function of the watersplitting enzyme system Y, because this kinetics disappeared in Tris-washed chloroplasts (s. Fig. 2) as well as in hydroxylammonim-salt-treated algae [25]. That means, the electron donor D_1 responsible for the fast kinetics of the Chl-a_{II}^+-reduction functionally interconnects *in vivo* the watersplitting enzyme system Y with the reaction center of system II.

The disappearance of the fast "undetected" Chl-a_{II}^+-reduction by Tris- or hydroxylammonium-treatment could be caused either by the modification of the redox properties of D_1 (similarly as the transition high → low potential form of cytochrome b 559, s. ref. 26) or by a functional disconnection of D_1 from Chl-a_{II} and (or) the watersplitting enzyme system Y. In the first case D_1 would stay in an oxidized form which is unable to become reduced by the watersplitting enzyme system Y, because of the low redox potential of the modified D_1. Therefore, the fast "undetected" Chl-a_{II}^+-reduction as well as the oxygen evolution would disappear, in agreement with the experimental data. This would also occur in the second case. However, the decrease of ΔA_0(Chl-a_{II}) in Tris-washed chloroplasts by electron donors (s. Fig. 4) indicates that the functional connection Chl-$a_{II} - D_1$ is not seriously modified by Tris-treatment. This favours the first alternative.

Taking into account the new results, the reaction scheme for the donor side of system II given in Fig. 4 of ref. 4 has to be extended. Generally two types of arrangements of the electron carriers on the donor side are possible: a series (s. Fig. 5, top) or a branched (s. Fig. 5, bottom) electron transfer chain.

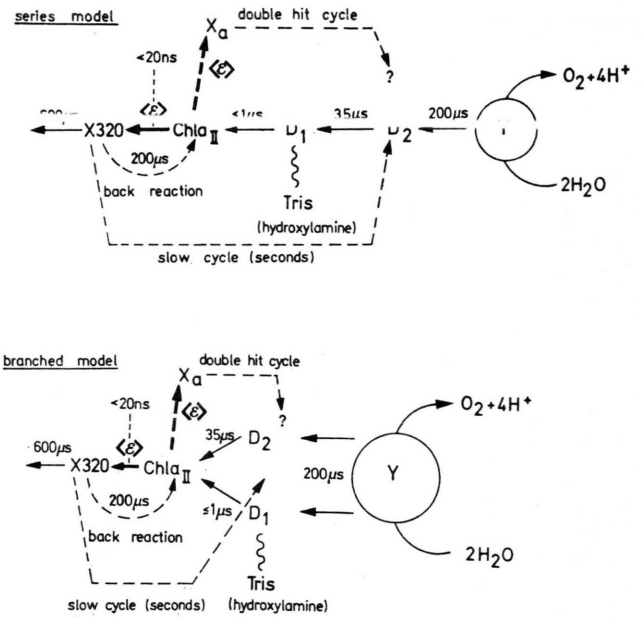

Fig. 5. Reaction schemes of system II electron transport. Top: series electron transport chain of the donor side. Bottom: branched electron transport chain of the donor side. For explanation s. text.

For the sake of simplicity, in both schemes D_1 is assumed to be a one-electron redox carrier, so that the amplitude of the fast electron transfer reaction is proportional to the degree of D_1-reduction. The chemical nature of this donor D_1 as well as of the other carrier D_2 is unknown. At the present stage of knowledge it is impossible to decide about the mode of functional (series or branched model) and structural organization of the donor side of system II.

The location of the 200 μs component ($\tau_{1/2} = 140 - 220$ μs) of the Chl-a_{II}-recovery is not yet clarified. On the basis of measurements of delayed luminescence [9] this kinetics seem to reflect a direct charge recombination at the reaction center. Kinetic data on Tris-washed chloroplasts support this assumption (s. ref. 7, 9). On the other hand, in normal chloroplasts the extent of the 200 μs kinetics is too large to be explainable exclusively by charge recombination at C_{II+}^-, because the quantum yield of the overall system II electron transport was found to be ~ 1 [10]. Therefore, besides a 200 μs-recombination kinetics there should exist another reaction on the donor side of system II with a similar half life time, as is indicated in the schemes of Fig. 5.

The postulation of a double hit probability leading with an efficiency of approx. 17% to the transformation of system II reaction centers with a fast Chl-a_{II}-recovery kinetics into slower ones requires the introduction of a further postulate.

As the regeneration of the primary electron acceptor known to be a special plastoquinone [27–29], called X 320, appears to be rather slow in comparison to the flash duration [29, 30] a significant double hit probability for the Chl-a_{II}-photooxidation is only possible, if a second electron acceptor is available. This component will be designated as X_a (auxilliary primary electron acceptor, s. ref 31) because it acts only under special conditions and with a rather low photochemical quantum yield ($\Phi^* = 0.17$ *versus* $\Phi \approx 1$ of the normal charge transfer at the reaction center in the state C_{II}). Very recently the existence of an endogeneous compound, which is able to act as a second primary electron acceptor under special conditions, has also been inferred by Duysens group on the basis of delayed fluorescence measurements [12]. Thus, a second type of photochemical charge transfer seems to occur in system II under a very specific functional state of the reaction center. However, this photoreaction does not produce utilizable redox equivalents, because neither the average oxygen yield per flash (G. Renger, unpublished results), reflecting the number of utilizable holes, nor the total amplitude ΔA_0 (Chl-a_I), reflecting the number of utilizable electrons of system II, show a similar dependency on the actinic flash intensity as ΔA_0(Chl-a_{II}) (s. Fig. 2). That means, the above mentioned photoprocess does not significantly contribute to the probability β of double oxidation of the water-splitting enzyme system Y (s. ref. 22).

The present model offers also a simple kinetical explanation for the oscillation pattern in a flash train of the amplitudes of delayed fluorescence in the μs-range, firstly observed by Zankel [32]. In terms of the back reaction model of Lavorel (for rev. s. ref. 8) the delayed fluorescence in the μs-range is of the "leakage type" and its intensity is given by:

$$L(t) = \Phi_F(t) \cdot \nu \cdot e^{-\frac{\Delta E(t)}{RT}} \cdot [C_{II+}^-(t)], \qquad (2)$$

where $\Phi_F(t)$ = fluorescence quantum yield, ν = frequency factor and $\Delta E(t)$ = activation energy of the internal back reaction leading to formation of an exciton $\langle\varepsilon\rangle$: $C_{II+}^- \rightarrow C_{II} + \langle\varepsilon\rangle$; $[C_{II+}^-(t)]$ = concentration of reaction centers in the state C_{II+}^- ($\triangleq X\,320^- \cdot$ Chl-a_{II}^+).

According to Eqn (2) an oscillatory pattern of $L(t)$ in a flash train resembling that of the oxygen evolution can be explained by an influence of the S_i-states of the watersplitting enzyme system Y exerted either energetically on the activation energy $\Delta E(t)$ or kinetically on the concentration $[C_{II+}^-(t)]$.

The energetical aspects have been already discussed in ref. 8, the kinetical effect can be brought into consideration on the basis of the present results. The hypothetical fast rereduction of Chl-a_{II}^+ causes a rapid decay of the precursor state C_{II+}^- of delayed fluorescence in $\leqq 1$ μs, so that in the range above a few microseconds the concentration of $C_{II+}^-(t)$ should be linearly correlated with the "detected" amplitude ΔA_0(Chl-a_{II}). Therefore, neglecting in a first approximation a possible influence of the S_i-states on the activation energy $\Delta E(t)$, the amplitudes of ΔA_0(Chl-a_{II}) and of the delayed fluorescence intensity in the range of above a few microseconds should be linearly interrelated to each other.

In Fig. 6 the experimental data of Zankel [32] and of Duysens *et al.* [11] giving the intensity of delayed fluorescence at 90 μs and 20 μs, respectively, after a flash are compared with the "detected" initial amplitudes ΔA_0(Chl-a_{II}) depicted in Fig. 3 of the present paper. The qualitative correspondence is obvious. By a combination of the present model and Kok's model [22] with $[S_0] = 0.25$; $[S_1] = 0.75$; $[S_2] = [S_3] = 0$ for dark adapted algae and chloroplasts and $\bar{\alpha} = 0.1$ and $\beta = 0$ the data can be explained by the assumption that the normalized amplitude a_2 (s. Eqn (1)) of the fast "undetected" Chl-a_{II}-re-

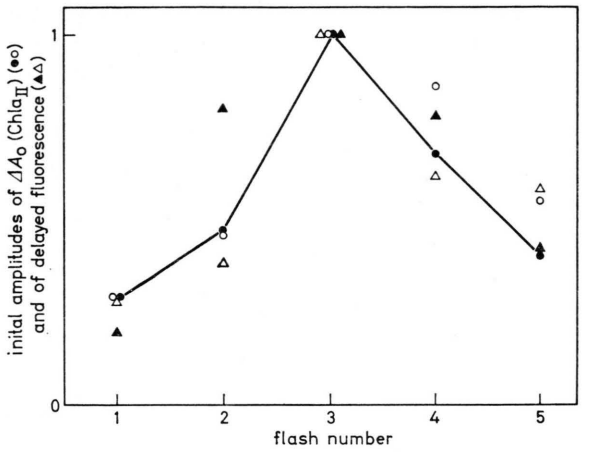

Fig. 6. Amplitudes of the absorption change at 690 nm, ΔA_0(Chl-a_{II}), and of delayed fluorescence intensity in the μs-range as a function of the flash number in a train. Experimental data for ΔA_0(Chl-a_{II}) are from Fig. 3 of the present paper (○) and for delayed fluorescence are redrawn from Fig. 4 of ref. 32 (△) and from Fig. 8 of ref. 11 (▲), respectively. The curve connects the theoretical values (●), s. text. The data are normalized for the corresponding values at the 3rd flash in the train.

covery kinetics depend on the S-states in the following way: $a_2(S_0) = 0.86$, $a_2(S_1) = 0.84$, $a_2(S_2) = 0.65$ and $a_2(S_3) = 0.05$. That means, according to the reaction schemes of Fig. 5 the donor D_1 is predominantly reduced, when the watersplitting enzyme system Y is in the states S_0, S_1 or S_2, but becomes highly oxidized, when system Y attains the state S_3. However, an oxidation degree of D_1 of 14% is not easily to rationalize for the state S_0. Therefore, the reduction kinetics of system Y attains the state S_3. However, an oxidation degree of D_1 of 14% is not easily to rationalize for the state S_0. Therefore, the reduction kinetics of Chl-a_{II}^+ might not be exclusively controlled by the redox level of D_1, but other factors (structural changes) could also interfer. Recently, a hypothetical mechanism for the photosynthetic water oxidation has been proposed [33]. According to this molecular model, the formation of "crytoperoxid" (complexed peroxide which is not accessible to hydrolysis into free H_2O_2) in the watersplitting enzyme system Y would be the essential step for the disappearance of the fast kinetics ($\leqq 1$ μs) of Chl-a_{II}^+-reduction. However, it is premature to make further speculations.

The existence of a fast electron transfer ($\leqq 1$ μs) from D_1 to Chl-a_{II}^+ readily explains the high photochemical quantum yield of system II, because the back reaction with a half time of approx. 200 μs leading to the dissipation of electrons and holes is slow in comparison to the above mentioned reaction between Chl-a_{II}^+ and D_1. The present results indicate that the stabilization of the primary photoproducts of the reaction center of system II is achieved by the very rapid withdrawing of the primary holes from Chl-a_{II}^+ rather than by a fast removal of the primary electrons from X 320$^-$.

Recently, Sauer and coworkers [34, 35] discovered EPR-signals which were ascribed to reflect the electron transfer reaction of a component located in the pathway between Chl-a_{II} and the watersplitting enzyme system Y. Within the framework of the reaction schemes of Fig. 5 the EPR-signals IIvf and IIf (s. ref. 34, 35) can be assumed to indicate the reactions of the component D_2. This will be discussed – together with the reaction sites of different system II electron donors – in a forthcoming paper. The present data have shown that the reaction mechanism of the oxidizing side of system II appears to be complex. Further experiments are required to clarify the details and to provide a direct experimental proof for the mechanism presented here, especially for the existence of a very fast ($\leqq 1$ μs) Chl-a_{II}^+-reduction time.

Note added in proof

After the manuscript has been submitted for publication, we became aware of new data of Haveman and Mathis published in Biochim. Biophys. Acta **440**, 346 [1976]. They showed that in dark adapted normal chloroplasts a double flash group does not lead to an appreciable formation of Chl-a_{II}^+-radicals with a lifetime longer than 60 μs, whereas in Tris-washed or acid-treated chloroplasts (procedures leading to impairment or interruption of the functional connection between Chl-a_{II} and the watersplitting enzyme system Y) the generation of Chl-a_{II}^+ with well resolved half life times of 100 – 200 μs can be observed. These results are in complete agreement with our present data and with the proposed model which predicts rather small amplitudes of the slow components of the Chl-a_{II}-recovery in normal chloroplasts when the watersplitting enzyme system Y attains the states S_0, S_1 or S_2 (the condition of the above mentioned measurements, s. ref. 22), but larger amplitudes of the slower kinetics in treated chloroplasts.

Appendix

The derivation of Eqn (1) is based on the hypothesis that there exists a fast phase of the rereduction kinetics of Chl-a_{II}^+ which is not detectable by our measuring device.

The first exciton reaching the reaction center in the state C_{II} leads to the normal charge separation: $X\,320 \cdot \text{Chl-a}_{II} \xrightleftharpoons{\varepsilon} X\,320^- \cdot \text{Chl-a}_{II}^+$. The quantum yield Φ of this process is close to unity [10]. If a_1 and a_2 denote the fractions of reaction centers C_{II} characterized by slow (35 μs and 200 μs) and fast ($\leqq 1$ μs) Chl-a_{II}-recovery kinetics, respectively, then after a few microseconds a_1 of the centers remained in the state $X320^- \cdot \text{Chl-a}_{II}^+$, whereas a_2 reach the state $X320^- \cdot \text{Chl-a}_{II}$. Furthermore, it is assumed that by a second exciton centers in the state $X\,320^- \cdot \text{Chl-a}_{II}$ are transformed into

$$(X_a^-)\,X\,320^- \cdot \text{Chl-a}_{II}^+$$

with an efficiency Φ^* and that in this state Chl-a_{II}^+ becomes rereduced *via* the "detected" slower kinetics (35 μs and 200 μs). Thus, for the fractions a_1 and a_2 of the reaction centers C_{II} a different saturation curve arises for the "detected" amplitudes $\Delta A_0(\text{Chl-a}_{II})$.

For the sake of simplicity of the analysis the photosynthetic units of system II are assumed to act as isolated entities with respect to exciton migration [36] and the absorbancy of the samples is neglected. Then, for the fraction a_1 one obtains (s. ref. 37) the normal saturation curve:

$$a_1 \frac{\Delta A_0(\text{Chl-a}_{II})}{\Delta A_0(\text{Chl-a}_{II})_{max}} = a_1(1 - e^{-\varrho}), \quad \text{(A 1)}$$

where ϱ denotes the average number of excitons per flash and per reaction center C_{II} and the photochemical quantum yield $\Phi = 1$.

On the other hand, the amplitude of Chl-a_{II}^+ of the fraction a_2 of system II reaction centers becomes detectable only after transformation by a second photoprocess with a quantum yield Φ^*. As the first exciton reaching C_{II} does not lead to the transformation, only centers of system II receiving two or more excitons per flash can be taken into account. The probability of centers being not transformed after $m \geqq 2$ excitons into the slower "detectable" form of Chl-a_{II}^+ is given by $(1 - \Phi^*)^{m-1}$. Thus, according to the Poisson distribution of excitons it results:

$$a_2 \cdot \frac{\Delta A_0(\text{Chl-a}_{II})}{\Delta A_0(\text{Chl-a}_{II})_{max}} = a_2 \sum_{m=2}^{\infty} [1 - (1 - \Phi^*)^{m-1}] \frac{\varrho^m}{m!} e^{-\varrho}. \quad \text{(A 2)}$$

The summation gives:

$$a_2 \cdot \frac{\Delta A_0(\text{Chl-a}_{II})}{\Delta A_0(\text{Chl-a}_{II})_{max}} = a_2 \left[1 - \frac{1}{1 - \Phi^*}(e^{-\Phi^* \cdot \varrho} - \Phi^* \cdot e^{-\varrho})\right]. \quad \text{(A 3)}$$

Taking together, one obtains with $a_1 + a_2 = 1$:

$$\frac{\Delta A_0(\text{Chl-a}_{II})}{\Delta A_0(\text{Chl-a}_{II})_{max}} = 1 - a_1 \cdot e^{-\varrho} - \frac{a_2}{1 - \Phi^*}(e^{-\Phi^* \cdot \varrho} - \Phi^* \cdot e^{-\varrho}). \quad (1)$$

According to Eqn (1) the experimental results of Fig. 2 are best fitted with $a_1 = 0.4$; $a_2 = 0.6$ and $\Phi^* = 0.17$.

It should be emphasized that the time ratio between flash duration and the "undetected" Chl-a_{II}-recovery modifies Eqn (A 3). However, it was shown (unpublished results) that under our conditions ($\tau_{flash} = 20$ μs *versus* $\tau_{1/2} \leqq 1$ μs of Chl-a_{II}-recovery) this effect can be neglected.

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