INVESTIGATION OF THE REACTIONS OF CHLOROPHYLL-a_{II} IN CLASS II CHLOROPLASTS UNDER REPETITIVE DOUBLE FLASH GROUP EXCITATION

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Received 19 April 1979

1. Introduction

The primary photochemical event at the reaction centers of photosystem II was suggested to be the electron transfer from an electronically excited special chlorophyll *a* (designated as Chl-a_{II}) to a special plastoquinone molecule [1–3], referred to as *X*-320 [2]. *X*-320 acts as a one-electron redox component only, whose plastosemiquinone form becomes reoxidized by a secondary plastoquinone acceptor designated B (or R) [4,5] via a dark reaction with a 600 μs half-life [2,6]. The recovery of Chl-a_{II} was found to be significantly faster (characterized by a multiphasic kinetics) in the micro- and submicrosecond range [7–9]. Accordingly, the photochemical turnover of the photosystem II reaction centers should be limited by the 600 μs reoxidation kinetics of *X*-320$^-$. However, former measurements under repetitive flash groups of the ΔA_{690}, reflecting the reversible bleaching of Chl-a_{II}, indicated that the amplitude of the absorption change caused by the second flash of the group is nearly the same as that induced by the first flash at a dark time between the flashes which is smaller than the half-life of reduced *X*-320$^-$ [1]. This result could be explained either by the action of Chl-a_{II} as a sensitizer molecule [1] or by the existence of a further electron acceptor available for the photooxidation of Chl-a_{II}. However, as the analysis was restricted to the 200 μs kinetics, which reflect only a minor fraction of the total Chl-a_{II} turnover, an unambiguous conclusion cannot be drawn from these results. Recently, different lines of indirect evidence led to the suggestion that Chl-a_{II} is photoactive under conditions where X 320 is functionally blocked in its reduced state [10–13]. These results favour the existence of an acceptor component, which is able to support the photooxidation of Chl-a_{II}, when *X*-320 remains reduced.

In order to clarify the reaction pattern of Chl-a_{II} with respect to the functional state of the acceptor side, light-induced ΔA_{690} were measured under double flash group excitation.

The results obtained provide further evidence for the existence of a primary electron acceptor of photosystem II other than *X*-320.

2. Materials and methods

Class II chloroplasts were prepared from market spinach by the method in [14], except for the addition of 10 mM ascorbate during grinding. For storage in liquid nitrogen 5% dimethylsulfoxide was added. The maximal average oxygen yield per flash after rethawing of the frozen chloroplasts was practically the same as that of freshly prepared chloroplasts.

The standard reaction mixture contained: chloroplasts (5 μM chlorophyll), 300 μM $K_3[Fe(CN)_6]$, 10 mM KCl, 2 mM $MgCl_2$ and 20 mM *N*-tris(hydroxymethyl)methylglycine (tricine)–NaOH at pH 7.5 or 20 mM morpholinoethanesulfonat (MES)–NaOH at pH 6.0. Other additions as indicated in the figure legends.

The absorption changes were recorded with a repetitive flash photometer by the application of

This work has been presented in part at the Biophysik-Tagung Ulm, 1–4.10.1978

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14 MHz-modulated detecting light beam [15] in order to eliminate fluorescence artefacts due to the actinic flashes. In order to improve the signal/noise ratio 8192 signals were averaged per measurement in a NIC 1170, but the sample was changed after each 1024 flashes. Photosynthesis was excited with double flash groups at a group repetition rate of 5 Hz, the duration of each flash was ~20 μs. The flash light passed a Schott-filter BG 23/3. The optical pathlength was 20 mm, the intensity of the detecting beam $\leq 30\ \mu W/cm^2$ and its optical bandwidth 10 nm. In order to suppress fast kinetics due to photosystem I turnover, far-red background illumination was applied [16] with an intensity of 290 $\mu W/cm^2$.

3. Results

Figure 1 shows a typical time course of the ΔA_{690} induced by repetitive double flash groups in normal class II chloroplasts at pH 7.5. The bleaching caused by the first flash (duration 20 μs) decays with half-time of 100 μs order. Under our experimental conditions these μs-decay kinetics reflect the recovery of Chl-a_{II}, since the reduction of P-700 in the μs range is eliminated by the application of strong far-red light [16]. 200 μs after a 20 μs flash Chl-a_{II} has recovered substantially [7–9], but the acceptor X-320 remains

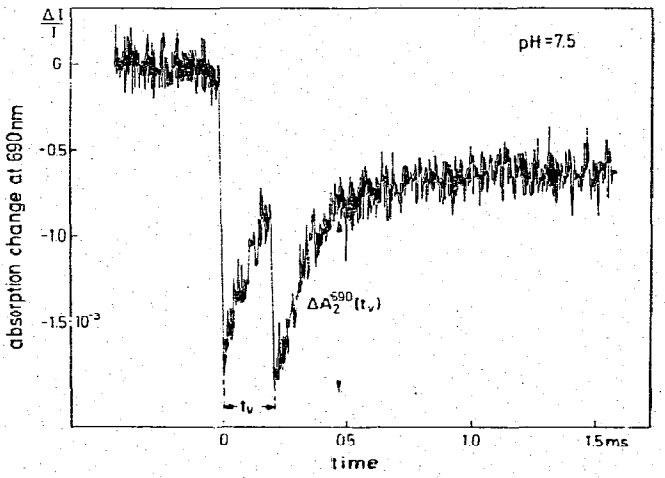

Fig.1. ΔA_{690} induced by repetitive double flash groups as a function of time in isolated class II chloroplasts at pH 7.5. The time between the flashes of a group was 200 μs, other experimental conditions as in section 2.

predominantly (> 70%) reduced, because of its comparatively slow reoxidation kinetics [2]. Accordingly, a second flash fired 200 μs after the first flash should induce only a rather small ΔA_{690} if X-320 is assumed to be the only primary acceptor component available in photosystem II and the 690 nm bleaching reflects the photooxidation of only Chl-a_{II}^+. The data of fig.1 indicate that this is not the case, in agreement with the results in [1]. The second flash causes nearly the same extent of maximal bleaching as the first one. If one accepts that in the presence of strong far-red light the μs kinetics reflect exclusively the reduction of photooxidized Chl-a_{II}^+, then the amplitude observed in fig.1 amounts to $\geq 40\%$ of the total Chl-a_{II}, based on the molar extinction coefficient difference $\Delta\epsilon$(Chl-a_{II}^+/Chl-a) at 690 nm being the same ($6.4 \times 10^4\ M^{-1}\ .cm^{-1}$) as that for P-700 oxidation at 703 nm [17] and 1 Chl-a_{II}/500 chlorophylls. The percentage would be even higher on the basis of the extinction coefficient difference for the maximal bleaching in the red due to Chl-a^+ formation in vitro in CH_2Cl_2 [18]. Taking into account the flattening effect [30], the data of fig.1 reflect the turnover of ~50% of the total Chl-a_{II} content. As the time resolution of the present measurements was limited by the flash duration of ~20 μs (the contribution of faster kinetics to the Chl-a_{II}^+ reduction [8,9] escaped our detection) the total extent of Chl-a_{II}^+ formation cannot be observed. Accordingly, one cannot decide whether the absorption change caused by the second flash of the group represents the fraction of the photosystem II reaction centers which are 'reopened' by the oxidation of X-320$^-$. This would involve the additional assumption that the reduction by the electron donor (donors) for Chl-a_{II}^+ in the 'reopened' centers occurs practically exclusively via an overall kinetics of 100 μs order. To test this hypothesis thoroughly the initial amplitude of the absorption change in the μs range, caused by the second flash [$\Delta A_2^{690}(t_v)$] was measured as a function of the time t_v between the flashes of a group. The results depicted in fig.2 indicate that the recovery of $\Delta A_2^{690}(t_v)$ is characterized by a 100 μs half-rise time. This is at least 5-times faster than the reoxidation kinetics of X-320$^-$ and the dependency on t_v of the initial amplitude of X-320 (measured at 335 nm) induced by the second flash of repetitive double flash groups [2,6]. Therefore the results are not in agreement with

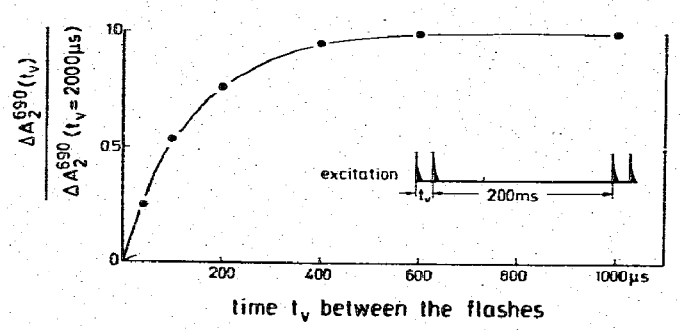

Fig.2. Initial amplitude of the ΔA_{690} induced by the second flash $[\Delta A_2^{690}(t_v)]$ as a function of the time t_v between the first and second flash of repetitive double flash groups in isolated class II chloroplasts at pH 7.5.

the above-mentioned hypothesis. As another type of experimental test, double flash group measurements were performed at pH 6.0, where the total amplitude of the slower µs kinetics of the Chl-a_{II} recovery were found to be significantly greater under repetitive flash excitation [19]. The results depicted in fig.3 (top) indicate that at pH 6.0 generally the same pattern arises as that at pH 7.5, but the total extent of the absorption changes increased by 50% (in some experiments an increase of $\leqslant 70\%$ is observed). If one assumes the $\Delta\epsilon$(Chl-a_{II}^+/Chl-a_{II}) to be invariant with pH 6.0–7.5, then the results lead to the conclusion that $\geq 75\%$ of the total Chl-a_{II} can be photooxidized

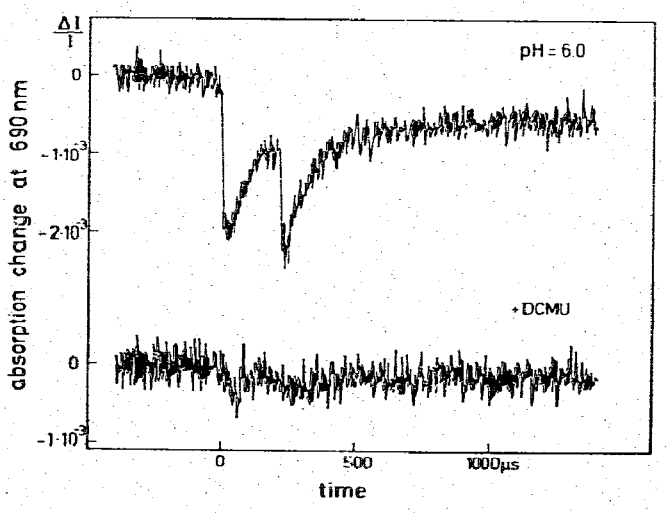

Fig.3. ΔA_{690} induced by repetitive double flash groups as a function of time in isolated class II chloroplasts at pH 6.0 in the absence (top) and presence of 2 µM DCMU (bottom). Excitation conditions as in fig.1.

by a flash fired 200 µs after a 20 µs flash. Accordingly, the results of fig.1–3 cannot be explained by 600 µs reoxidation kinetics of X-320$^-$. If one presupposes these kinetics to be valid for all reaction centers [2,6,20], 3 alternative modes of explanation can be considered:

(i) Chl-a_{II} itself does not become photooxidized, but acts only as a sensitizer for the photochemistry at the reaction centers, whose bleaching at 690 nm is independent of the functional state of X-320;

(ii) The ΔA_{690} caused by the second flash reflects the reaction of a Chl-a species other than Chl-a_{II};

(iii) There exists an additional component (other than X-320) which is able to act as a primary electron acceptor for the photooxidation of Chl-a_{II}

Despite lack of direct proof for the oxidation of Chl-a_{II}, the simple sensitizer model (which was earlier shown to be very improbable [7,31]) is hardly reconcilable with the dependence of the recovery kinetics of the ΔA_{690} on the functional state of the water-splitting enzyme system Y [8–10] and on the internal pH of the thylakoids [19]. Furthermore, the complete suppression under repetitive excitation of the ΔA_{690} by DCMU, shown in the bottom of fig.3, cannot be explained by the sensitizer model, because the primary reactions of photosystem II [21,22] and even oxygen evolution were found to be highly resistent to DCMU [23–25]. Hence, a sensitizer model does not consistently explain the present results. Analogously, the reaction of an unspecific Chl-a species, which is not involved in the photochemical processes at the reaction centers can be excluded on the basis of the DCMU experiments of fig.3, bottom.

In order to corroborate that the ΔA_{690} induced by the second flash of the group at t_v = 200 µs is caused by Chl-a_{II}, the same type of experiment was performed at 703 nm. The results are shown in fig.4. The kinetic pattern at 703 nm significantly differs from that observed at 690 nm. The µs kinetics are markedly reduced for both flashes and the amplitude of the slower kinetics, which are ascribed to the reduction of photooxidized P-700$^+$, become significantly enhanced for the first flash of the group, while the second flash does not give rise to a pronounced absorption change. These results confirm that the ΔA_{690} with a recovery kinetics of ~100 µs is due to the turnover of Chl-a_{II}.

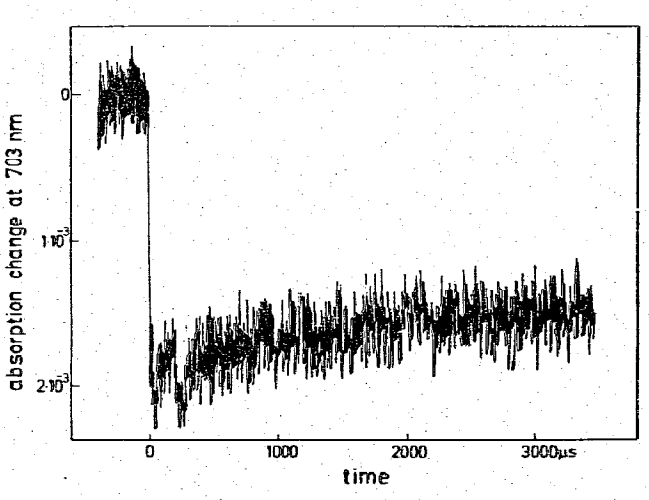

Fig.4. ΔA_{703} induced by repetitive double flash groups as a function of time in isolated class II chloroplasts at pH 7.5. Excitation conditions as in fig.1.

The results presented in fig.1–4 lead to the conclusion, that there exists a component other than X-320, which is able to act as an electron acceptor for the photooxidation of Chl-a_{II}, when X-320 remains functionally blocked in its reduced state. This component will be referred to as X_a. Furthermore, on the basis of the results of fig.3 it can be estimated, that most of the reaction centers of photosystem II (if not all of them) contain the component X_a.

4. Discussion

If one agrees that the semiquinone form of the photosystem II acceptor X-320 of all reaction centers becomes reoxidized with $\tau_{1/2} = 600$ µs ([2,6]; for a more refined analysis [20]) and that the bleaching at 690 nm reflects the photooxidation of Chl-a_{II} ([7,31]; present arguments), then the present results corroborate the existence of a redox component X_a, which is able to act as a primary electron acceptor during the photooxidation of Chl-a_{II}. Thus, the photochemical turnover of the reaction centers of system II does not become limited by the reoxidation of X-320$^-$. Accordingly, double hit processes can arise by excitation with flashes of a few µs duration (as discussed in [10–13]), because the recovery of Chl-a_{II} was found to be rather fast in chloroplasts with an intact watersplitting enzyme system Y [8,9]. The existence of a component X_a rises questions about the functional organization of the acceptor side of photosystem II reaction centers and the chemical nature of X_a. The latter remains completely unresolved, the former will be detailed in [26]. Very recently, on the basis of redox titration of the variable fluorescence and its rise kinetics [27,28] as well as of the extent of the flash-induced ΔA_{515} [29] the existence of two different types of photosystem II acceptor components has been derived. However, as our measurements were performed at rather high redox potentials (presence of $K_3Fe(CN)_6$) the present data are not influenced by the above-mentioned different redox behaviour and X_a cannot be identified with one of the acceptors discovered by the redox titrations.

Acknowledgements

The authors are very grateful to Professor Dr H. T. Witt for his stimulatory interest in this work. We also thank Mrs Hachtmann for technical assistance and Mrs A. Schulze for drawing the figures. The financial support by ERP-Sondervermögen is gratefully acknowledged.

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