Reduction kinetics of the photo-oxidized chlorophyll a_{II}^{+} in the nanosecond range

Measurements of the absorption changes at 688 nm under repetitive flash excitation

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The 688 nm absorption changes (ΔA_{688}), indicating the photochemical turnover of chlorophyll a_{II} (Chl a_{II}) have been investigated under repetitive laser flash excitation conditions in spinach chloroplasts. It was found that under steady state conditions about 50–60% of the photo-oxidized primary donor of Photosystem II (PS II), Chl a_{II}^{+}, becomes re-reduced with a biphasic kinetics in the nanosecond time scale with half-life times of about 50 ns and 400 ns. The remaining Chl a_{II}^{+} becomes re-reduced in the microsecond range.

Photosystem II *Chlorophyll a_{II}* *Electron transfer* *Reaction center*

1. INTRODUCTION

Photosynthetic water oxidation to O_2 requires the light-induced oxidation of a special chlorophyll *a* complex (Chl a_{II}) within the system II reaction center, and subsequent cooperative redox reactions that take place within the water-splitting enzyme system Y. Information about the mode of coupling between both units should be obtainable by measurements of the Chl a_{II}^{+} reduction kinetics.

The turnover of Chl a_{II}, induced by short flashes, can be monitored either directly by absorption changes or indirectly by fluorescence measurements. Negative absorption changes peaking around 690 nm (ΔA_{688}) were reported to reflect the photo-oxidative bleaching of Chl a_{II} [1,2], while positive absorption changes around 820 nm (ΔA_{820}) are inferred to indicate the transient Chl a_{II}^{+} cation radical formation [3].

If chloroplasts are selectively deprived of their oxygen evolving capacity (e.g., by Tris-washing) H_2O cannot function as ultimate electron donor to Chl a_{II}^{+}. Under repetitive flash excitation Chl a_{II}^{+} becomes then re-reduced by a back reaction with the semiquinone form of the primary plastoquinone acceptor, $X320^{-}$ [3–5]. Under these conditions the relaxation kinetics of ΔA_{690} and ΔA_{820} are practically identical [3,4] and coincide with those reflecting the $X320^{-}$ oxidation [5]. However, different and complex kinetics are observed in chloroplasts with intact system Y. Measurements of ΔA_{820} in dark-adapted chloroplasts led to the conclusion that Chl a_{II}^{+} becomes reduced predominantly via a reaction with a half-life of 25–45 ns [6,7], whereas a complex kinetics of ΔA_{690} was observed under repetitive flash excitation with components of 2–4 μs, 35 μs and 200 μs [8,9]. Unfortunately, the data are not directly com-

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parable due to the quite different excitation conditions (this problem has been discussed in detail in [10]). Indirect evidence from fluorescence measurements on *Chlorella vulgaris* [11] suggest that the Chl a_{II}^{+} reduction kinetics are dependent on the dark adaptation state (30 ns *vs* 400 ns half-life time after single and repetitive flash excitation, respectively).

The measurements of ΔA_{690} reported so far were limited in time resolution (1 μs) due to the problems caused by the fluorescence artifact. Therefore the development of new equipment was required in order to be able to analyze the questions arising for the interpretation of the ΔA_{690} and ΔA_{820} measurements. Our results indicate that under repetitive excitation with laser pulses the relaxation kinetics of ΔA_{690} are multiphasic with different components in the nanosecond and microsecond range. The data are discussed in the light of previously published data.

2. MATERIALS AND METHODS

The measurements of the absorption changes in the range of 670–710 nm were performed with a newly developed flash photometer, which drastically reduces fluorescence artifacts by a special setup of the measuring light beam and the detector. As light source we used a dye laser (Spectra Physics, type 375, dye: DCM) pumped by the 514 nm beam of a cw argon laser (Coherent Associates, Innova 90/5). The solid angle of light collection at the detector was drastically diminished by the use of a diaphragm with an aperture of 1 mm which was 1.5 m from the cuvette (the principle of this method is described in [12]). The photodetector was further protected by a narrow band interference filter. The details of the equipment are described in [13]. Photosynthesis was excited with pulses from a Q-switched frequency doubled Nd:YAG-laser (YG 441 from Quantel, λ = 532 nm, pulse energy about 10 mJ, pulse length: 3 ns).

The electrical bandwidth of the photodetector and the amplifier ranged from 100 Hz to 50 MHz. 512 signals were digitized in a Biomation 6500 and averaged in a Nic 1170. In order to eliminate a still remaining fluorescence artifact 512 fluorescence signals alone (without measuring light) were averaged after each experiment and subtracted from the original signal. For the measurement of the fluorescence signal the photodetector was illuminated by white light giving rise to the same photocurrent as the measuring light beam and the sample was illuminated by light of the same intensity and wavelength as the measuring beam. Class II- and Tris-washed spinach chloroplasts were prepared as in [14] and [15], respectively. The standard reaction mixture contained chloroplasts (5 μM chlorophyll), 10 mM KCl, 2 mM $MgCl_2$, 20 mM *N*-tris(hydroxymethyl)methylglycine (Tricine)–NaOH (pH 7.5) and 300 μM $K_3Fe(CN)_6$ as electron acceptor. The optical pathlength was 20 mm, the monitoring light intensity about 100 $\mu W/cm^2$, and optical bandwidth <1 nm. All experiments were carried out at room temperature.

3. RESULTS

Typical traces of absorption changes at 688 nm (ΔA_{688}) obtained in normal and Tris-washed chloroplasts under repetitive flash excitation are shown in fig.1 and in fig.2 on an extended time scale. The control signal (fig.1A,2,top) reveals characteristic relaxation kinetics in the nanosecond and microsecond range. However, in the first few nanoseconds after the actinic flash a very short signal is detected (decay <10 ns), which remains almost unaffected by DCMU-addition or Tris-washing of the chloroplasts. The interpretation of this signal is very complicated because the possible interference with an artifact cannot be excluded. Therefore this part of the signal will not be discussed here.

In order to show that the kinetics remaining after the first few nanoseconds in the control signal (fig.1A,2,top) really does reflect the recovery of Chl a_{II}, the effects of DCMU, of DBMIB plus far-red background illumination and of Tris-washing on the signal were analyzed. Fig.1B,2(bottom) shows that, after Tris-washing, the relaxation kinetics becomes highly retarded (the nanosecond and fast microsecond kinetics almost completely disappear) without effect on the total extent, while 3 μM DCMU significantly suppress the signal (fig.1C). On the other hand, the signal remains almost invariant to DBMIB plus far-red illumination. In the latter case, oxygen evolution is not suppressed because $K_3Fe(CN)_6$ was used as electron acceptor, while the PS I reactions are blocked

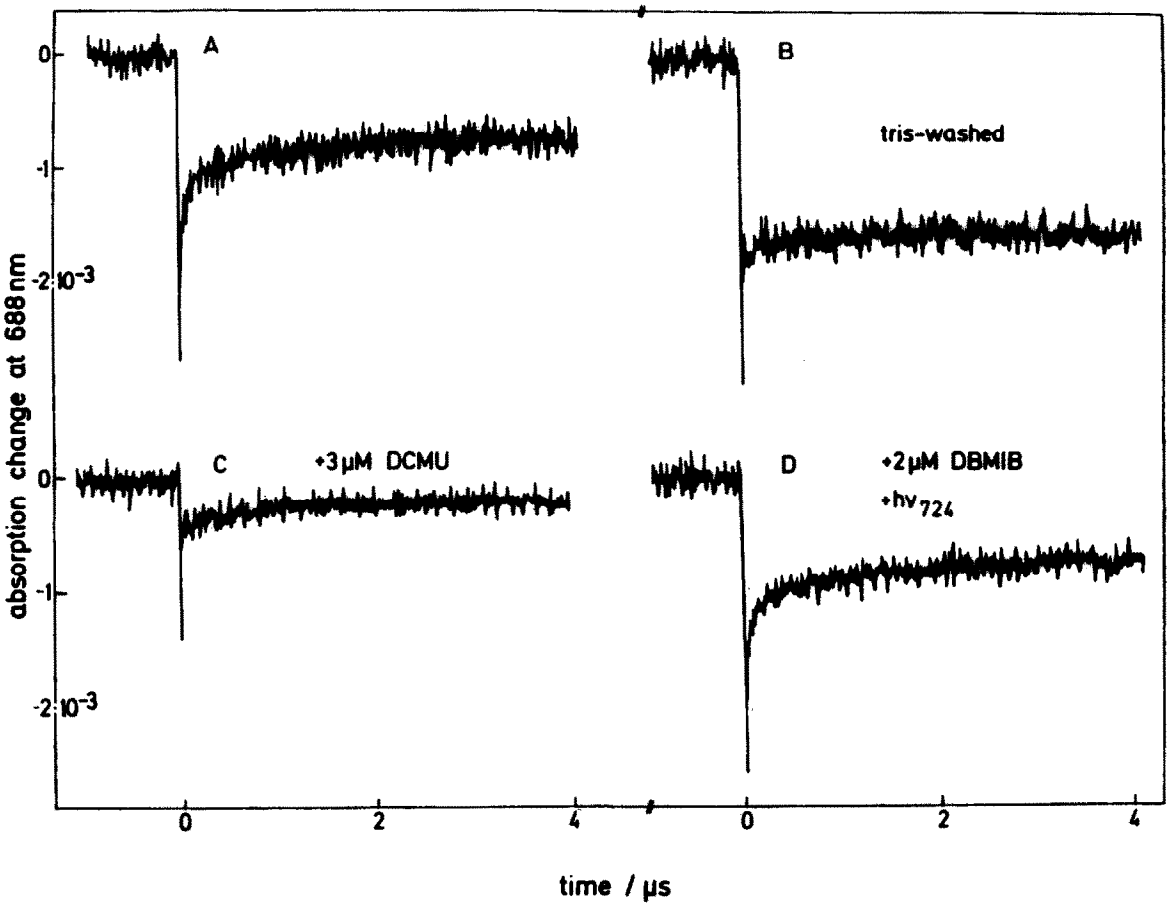

Fig.1. Absorption changes at 688 nm induced by repetitive 3 ns laser flashes (λ = 532 nm, repetition rate: 5 Hz) as a function of the time in isolated chloroplasts. (A) Normal chloroplasts, (B) Tris-washed chloroplasts, (C) normal chloroplasts in the presence of 3 μM DCMU, (D) normal chloroplasts in the presence of 2 μM DBMIB under illumination with 2 mW/cm far-red background light (λ = 724 nm).

[8,16–18]. These findings support the idea that the observed nanosecond and microsecond kinetics of ΔA_{688} reflect the turnover of Chl a_{II}. This conclusion is corroborated by measurements of absorption changes at 670 and 704 nm. The data presented in fig.3 clearly show that nanosecond recovery kinetics are hardly observed at these wavelengths, if the first few nanoseconds are not taken into account (vide supra). For an unambiguous assignment the difference spectrum of the amplitude differences, measured at 50 ns and 4 μs after the flash, respectively, has been determined as a function of wavelength. The data of fig.4 show a typical Chl a_{II}/Chl a_{II}^{+} difference spectrum [1,2], which is practically identical with the difference spectrum of the 130 μs kinetics in Tris-washed chloroplasts (not shown) due to the Chl a_{II}^{+} reduction by $X320^{-}$ via the back reaction [3–5]. Therefore it can be concluded that the signal ΔA_{688} observed in the control chloroplasts (fig.1A,2,top) reflects the light-induced turnover of Chl a_{II}. Accordingly the relaxation kinetics describes the reduction of Chl a_{II}^{+} by electrons ultimately extracted from the water-splitting enzyme system Y.

To be able to perform a kinetic analysis of the DCMU-sensitive signal, the slower kinetic phase has to be taken into account. Scanning on a larger time scale (not shown) reveals a complex pattern in the microsecond range with a dominating 15–40 μs kinetics, first discovered in [8] and smaller con-

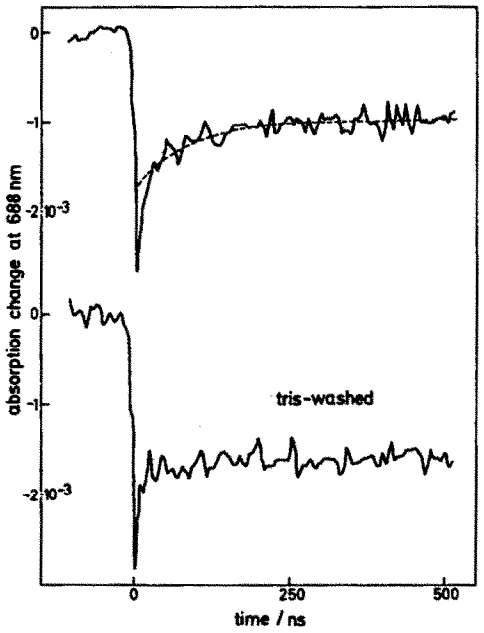

Fig.2. Absorption changes at 688 nm in normal (top) and Tris-washed chloroplasts (bottom) as a function of time. Same conditions as in fig.2A,B, but expanded time scale. For details see text.

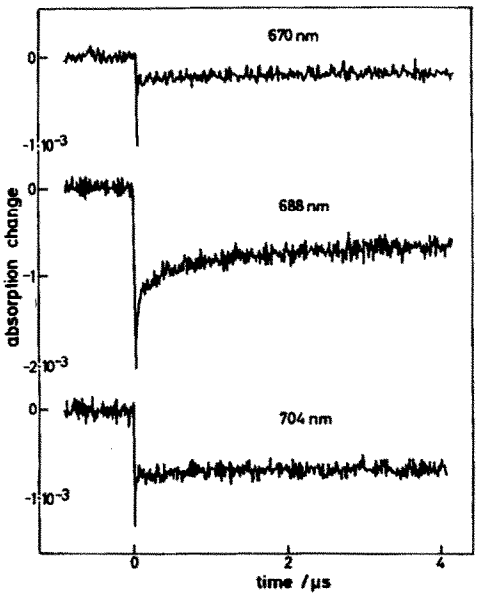

Fig.3. Absorption changes at 670, 688 and 704 nm as a function of time in normal chloroplasts.

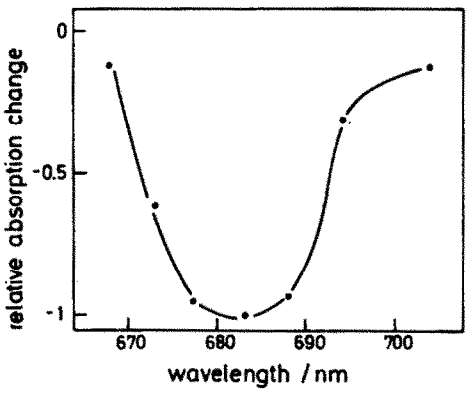

Fig.4. $\Delta A_{50ns} - \Delta A_{4\mu s}$ as a function of the wavelength. ΔA_{50ns}, $\Delta A_{4\mu s}$, amplitude of the absorption changes 50 ns and 4 μs after the actinic flash, respectively.

tributions of 3–7 μs and 200 μs components, previously reported in [9] and [1], respectively (an unambiguous separation of the 3–7 μs and the 15–40 μs kinetics is sometimes impossible). A semilogarithmic plot of the remaining components shows that the recovery in the nanosecond range cannot be described adequately by a single exponential kinetics. A satisfying description can be achieved if two exponential decay kinetics are assumed which differ by almost one order of magnitude. In this case values of half-lives of 50 ± 20 ns (about 1/3 of the total amplitude of ΔA_{688}) and 400 ± 150 ns (about 1/5 of ΔA_{688}) were found to give a good fit. The dotted line in fig.2 (e.g., top) represents a fit with $t_{1/2}$ = 50 ns (35% of ΔA_{688}) and $t_{1/2}$ = 500 ns (20% of ΔA_{688}). This analysis shows that 50–60% of the total amplitude of ΔA_{688} has a recovery kinetics in the nanosecond range.

An estimation of the difference extinction coefficient $\Delta\epsilon_{688}$ (Chl $a_{II}^{\dagger}$/Chl a_{II}) can be made on the basis of the extrapolated initial amplitude of ΔA_{688} (see fig.2,top) and measurements of the average oxygen yield per flash. Using a Clark-type electrode [19] a ratio of 360 ± 40 chlorophylls per PS II was found. This gives rise to a difference extinction coefficient of $\Delta\epsilon_{688}$ = 63 ± 10 $mM^{-1} \cdot cm^{-1}$, a value which closely resembles that for the bleaching maximum of Chl a_I at 704 nm [20].

4. DISCUSSION

This study is the first report of the recovery of the Chl a_{II} bleaching in the nanosecond time domain. The data reported here together with findings in [8,9] lead to the conclusion that the reduction kinetics of Chl a_{II}^{+} in chloroplasts with intact water-splitting enzyme system Y are rather complex under repetitive flash excitation, with half-life times of 50 ± 20 ns, 400 ± 150 ns and multiphasic kinetics in the microsecond range ($t_{½}$ = 3–7 μs, 15–40 μs and 200 μs). Our results are not directly comparable with previous findings at 820 nm leading to the discovery of the 30 ns kinetics [6] because these experiments were performed only with a single flash in dark-adapted systems (i.e., without oxygen-evolution in system Y), in contrast to our conditions, where the water oxidizing enzyme attains a steady redox state. The 400 ns and the microsecond kinetics were not observed in [6]. These differences can be explained by the assumption that the Chl a_{II}^{+} reduction is dependent on the redox state of system Y. Accordingly, the multiphasic kinetics might indicate the release of electrons out of the different S-states by an unknown mechanism through the electron carrier D.

A still unresolved problem remains the significance of the microsecond kinetics. Previous reports [8,9] and the present study show that under steady state conditions a significant fraction (40–50%) of the photo-oxidized Chl a_{II}^{+} becomes reduced in the microsecond range, whereas findings in [7] at 820 nm revealed a markedly smaller contribution (<25%). Further comparative experiments are required to clarify this point.

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