THE DETECTION OF INTRINSIC 320 nm ABSORPTION CHANGES REFLECTING THE TURNOVER OF THE WATER-SPLITTING ENZYME SYSTEM Y WHICH LEADS TO OXYGEN FORMATION IN TRYPSINIZED CHLOROPLASTS

G. RENGER and W. WEISS

Max Volmer Institut für Biophysikalische und Physikalische Chemie der Technischen Universität, Straße des 17. Juni 135, 1000 Berlin 12, Germany

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1. Introduction

The process of photosynthetic water oxidation can be schematically described by the well-known Kok-scheme as to occur via a 4-step univalent redox reaction sequence [1]. However, the chemical nature of the intermediary stages symbolized by S_i (index i indicates the number of oxidizing equivalents stored in the water splitting enzyme system Y) is completely unknown. On the basis of different lines of evidence manganese was postulated to play an essential functional role in the stabilization of the oxidizing redox equivalents produced by Chl-a^*_{II} (review [2]). Furthermore, on the basis of theoretical considerations about the bioenergetics of photosynthetic water oxidation cytochrome *b*-559 was discussed as a possible candidate to be involved in the reaction sequence [3]. Another component which was claimed to function in system Y is plastoquinone [4,5]. The latter suggestion coincides with recent findings indicating that the oscillation pattern of UV absorption changes in the range of 290–370 nm induced by a train of short saturating flashes in dark-adapted chloroplasts appears to be a superposition of 2 different oscillation patterns with periodicities of 2 and 4, respectively [6,7]. These periodicities are due to the intermediary accumulation of electrons and holes at the acceptor (special plastoquinone) and donor (system Y) side of system II.

Latest findings of Velthuys led to the conclusion that a component L, characterized by a broad maximum peaking around 320 nm, participates in the redox reaction sequence. Unfortunately, an unambiguous separation of the oscillation pattern could not be achieved in the afore-mentioned studies because differently treated samples were compared. This paper describes an attempt to circumvent this problem by using mildly trypsinized chloroplasts. As the electron transport of the acceptor side of system II in these chloroplasts was found to be interrupted [9,10], the binary oscillation of the UV-absorption changes can be supposed to be eliminated with $K_3[Fe(CN)_6]$ as electron acceptor. These data unambiguously confirm the participation of a component which undergoes transient UV absorption changes associated with the formation of molecular oxygen.

2. Materials and methods

Preparation of the chloroplasts was according to [11] and the mild trypsinization procedure as in [12].

The standard reaction mixture contained chloroplasts (10 μM chlorophyll), 2 mM $MgCl_2$, 10 mM KCl and 20 mM morpholinoethanesulfonate (MES)–NaOH (pH 6.5). Further additions are indicated in the figures.

Absorption changes were measured by a flash photometer [13] with a pulsed measuring light beam switched on for 20 ms to detect the flash-induced absorption change. After each train containing the required number of flashes (usually 4 flashes) the sample in the cuvette was automatically renewed (by magnetic valves) from a stock suspension of dark-adapted chloroplasts. 16–32 signals were averaged in a NIC 1170: excitation, xenon flashes (15 μs halfwidth, saturating intensity) passed through a Schott RG 1; optical pathlength, 10 mm; optical bandwidth, 2 nm; electrical bandwidth, 20 kHz.

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3. Results

To be able to analyze the oscillation pattern of absorption changes reflecting the turnover of the water-splitting enzyme system Y, the binary oscillations which are caused by the intermediary stepwise accumulation of 2 reducing redox equivalents at the secondary plastoquinone acceptor B have to be eliminated. Accordingly, mildly trypsinized chloroplasts were used because, on the basis of experiments performed under repetitive flash excitation, this procedure was inferred to interrupt selectively the functional connection between the primary plastoquinone acceptor X320 and B, simultaneously increasing the accessibility of reduced $X320^-$ to $K_3[Fe(CN)_6]$ drastically [9,14]. It is well known that DCMU effectively blocks the electron transfer from $X320^-$ to B [15]. Furthermore, in dark-adapted chloroplasts illuminated in the presence of DCMU with a single turnover flash $X320^-$ was shown to become reoxidized via an internal cycle [16,17] involving the intermediary redox state S_2 of the water-splitting enzyme system Y. The same effect should be observed in mildly trypsinized chloroplasts in the presence of benzylviologen (which is inert as electron acceptor of $X320^-$ for thermodynamical reasons) provided that this treatment selectively intersects the X320–B connection. The results in fig.1 confirm this to be the case. The data which quantitatively correspond with findings based on measurements of the electrochromic effect [17], confirm that trypsin is a powerful tool for the specific elimination of the X320–B electron transfer without seriously affecting the donor side. The same conclusion was drawn for trypsinized Tris-washed chloroplasts [18]. As the electron transfer from $X320^-$ to $K_3[Fe(CN)_6]$ only involves a one-electron transfer step, the binary oscillation pattern of the acceptor side is eliminated. Therefore, any oscillation pattern observed in mildly trypsinized chloroplasts in the presence of $K_3[Fe(CN)_6]$ should be a specific indicator

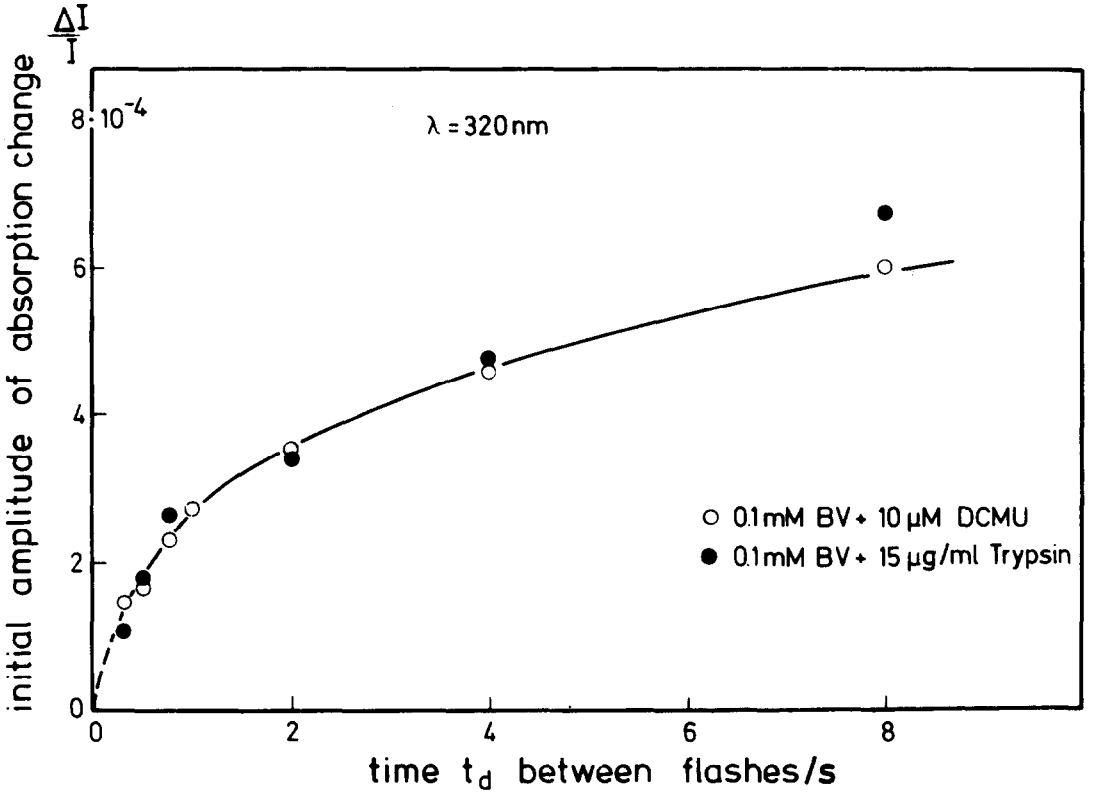

Fig.1. Initial amplitude of the absorption changes at 320 nm as a function of time t_d between the flashes in normal chloroplasts in the presence of 10 μM DCMU and in trypsinized chloroplasts in the absence of DCMU: chl, 15 μM; electron acceptor, 0.1 mM benzylviologen (BV).

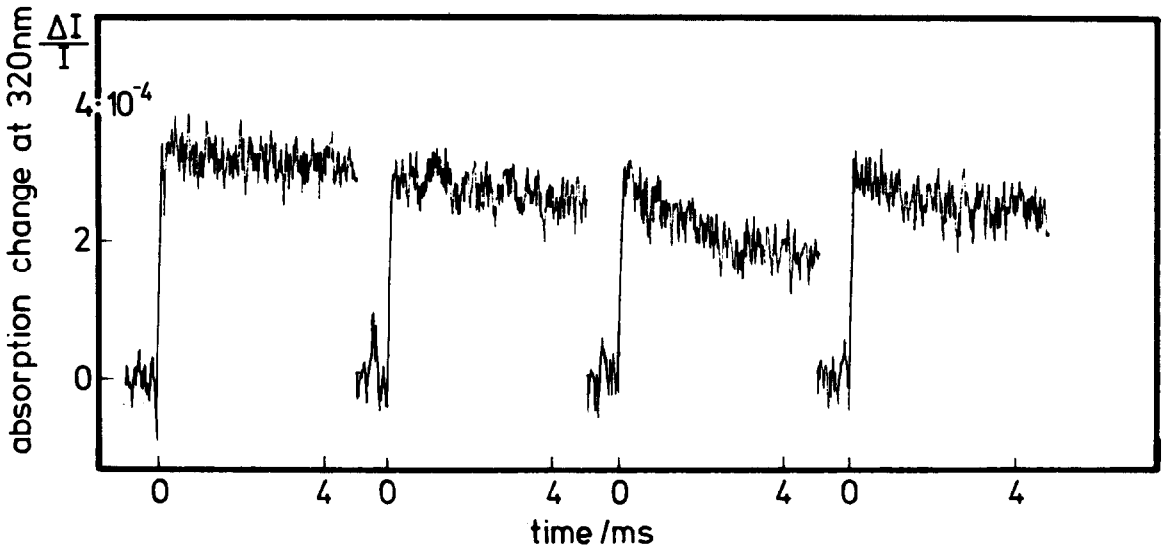

Fig.2. Absorption changes at 320 nm in dark-adapted trypsinized chloroplasts illuminated by a train of 4 flashes separated by a dark time t_d of 1 s: electron acceptor, 0.4 mM $K_3[FE(CN)_6]$; trypsin, 0.15 μg/ml chloroplasts suspension; 16 samples were averaged.

for reactions occurring at the donor side of system II. Absorption changes at 320 nm induced by a train of 4 flashes (each separated by a 1 s interval) in dark-adapted trypsinized chloroplasts in the presence of $K_3[Fe(CN)_6]$ are shown in fig.2. The signals exhibit a characteristic oscillation pattern in their relaxation kinetics. If one admits that the kinetics of the electron transfer from $X320^-$ to $K_3[Fe(CN)_6]$ is independent of the number of preilluminating flashes, the contribution due to the flash-induced turnover of X320 should be the same in each flash. Hence, any variation is expected to reflect reactions at the donor side. As the reoxidation kinetics of $X320^-$ in the presence of 0.4 mM $K_3[Fe(CN)_6]$ is rather slow, while the one-electron transfer steps within any intact water-splitting enzyme system Y are accomplished within 4 ms [19,20] the difference in the amplitudes ΔA_{320} (0) – ΔA_{320} (4 ms) should provide a rough measure of the extent of reactions taking place in system Y. The data depicted in fig.3 show an oscillation pattern which is characteristic for the oxygen evolution [1]. Accordingly, these absorption changes could reflect a reaction in system Y which is coupled with the formation of O_2. If this assumption is correct, the absorption changes should be abolished by the addition of Adry-agents, because these substances were found to attack selectively the redox states S_2 and S_3 of the water-splitting enzyme system Y [21]. The Adry-effect appears to be hardly affected by trypsinization of chloroplasts [12]. The results in fig.4 indicate that the oscillation completely vanishes in the presence of 0.3 μM 2-(3-chloro-4-trifluoromethylaniline)-3,5-dinitrothiophene (ANT 2p) which is known to be the most powerful Adry-agent [21]. A further test for the assignment of the oscillations of the 320 nm absorption changes to reactions coupled

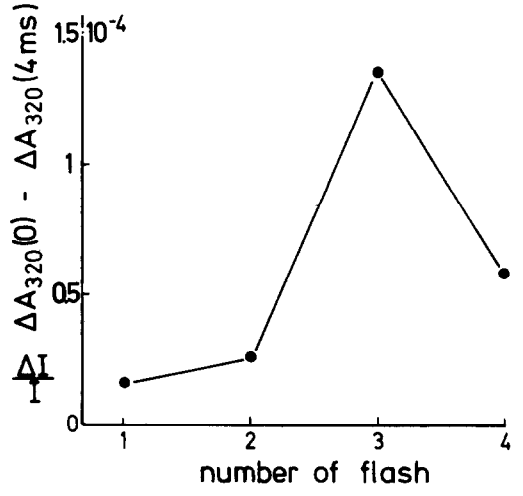

Fig.3. Difference of the initial amplitudes and the amplitudes 4 ms after flash excitation of absorption changes at 320 nm, ΔA_{320} (0) – ΔA_{320} (t_d), in dark-adapted trypsinized chloroplasts as a function of the flash number of the train. For experimental data see fig.2.

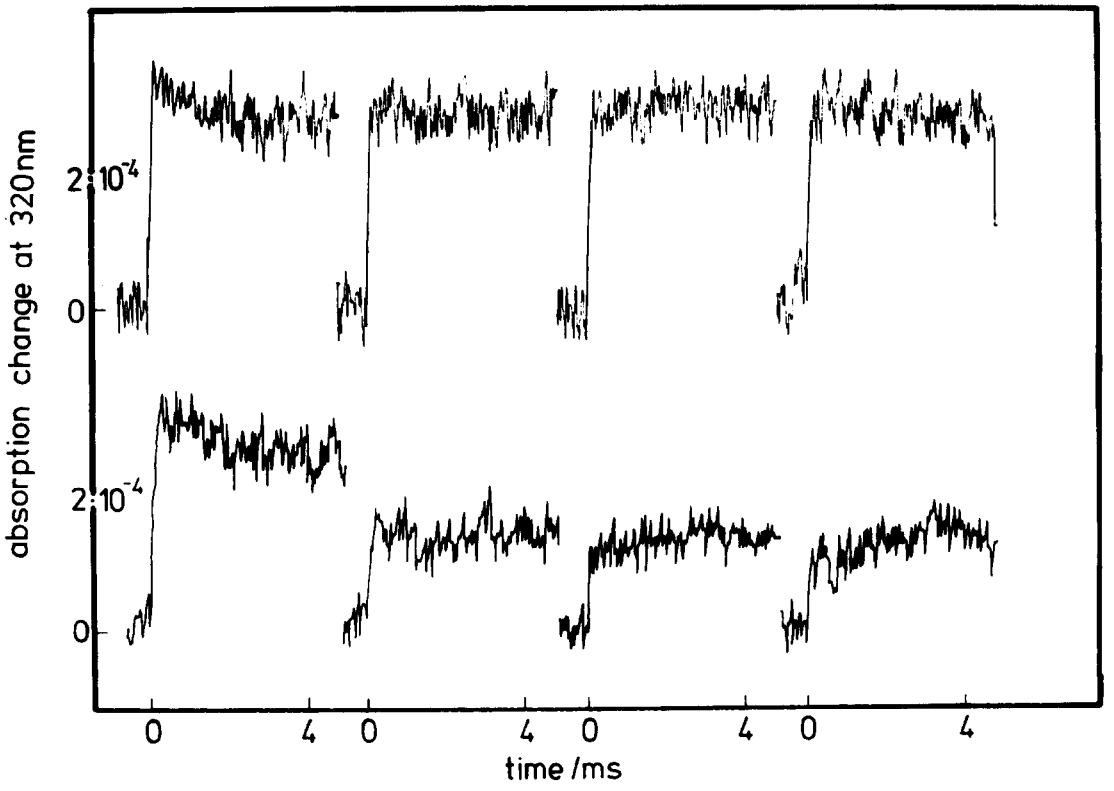

Fig.4. Absorption changes at 320 nm in dark-adapted trypsinized chloroplasts illuminated by a train of 4 flashes separated by a dark time t_d. Top: 0.4 mM $K_3[Fe(CN)_6]$; 0.3 μM ANT2p, t_d = 1 s. Bottom: 0.1 mM benzylviologen (BV), t_d = 2 s. 16 samples were averaged.

with oxygen formation in system Y is the analysis of the data obtained in trypsinized chloroplasts in the presence of benzylviologen. If the time between the flashes is sufficient for a significant extent of the back reaction (see fig.1), each flash induces the transitions $X320 \xrightarrow{e} X320^-$ and $S_1 \xrightarrow{\oplus} S_2$. Accordingly, the oscillation pattern observed in the presence of $K_3[Fe(CN)_6]$ should be absent in the presence of benzylviologen, if the difference $\Delta A_{320}(0) - \Delta A_{320}(4\,ms)$ really reflects a reaction intimately connected with the formation of O_2. The data of fig.4 (bottom) support this assumption. However, a superposition of 2 different types of X320 nm absorption changes is not sufficient to explain the observed pattern. A more detailed analysis will be given elsewhere (G. R., W. W., in preparation).

4. Discussion

These data indicate that the formation of oxygen in system Y is monitored by absorption changes at 320 nm. If one assumes that the extent of relaxation within 4 ms of the 320 nm absorption change induced by the first flash is due to the reoxidation of $X320^-$, the difference in extinction coefficients of the component participating in oxygen formation can be roughly estimated. Taking into account a flattening factor of 1.6 at 320 nm [23], 500 chl/system Y and 70% of all systems Y producing oxygen in the 3rd flash, one obtains from the data of fig.2 a value of $\Delta\epsilon_{320} \approx 4000–6000\ M^{-1} \cdot cm^{-1}$. Now the question arises about the chemical nature of the component reflected by the 320 nm absorption change which is involved in the reaction of oxygen formation in system Y. This component will be referred to as Y-320. Based on theoretical model studies [2,3] generally 2 alternative ideas should be considered:

(i) The component Y-320 could be a complex between a functional group (probably a manganese center) of system Y and O_2, which rapidly decays due to a ligand exchange reaction with water. In this case,

the 320 nm bleaching with a half-life of 1–2 ms would reflect this exchange reaction.

(ii) The transient 320 nm absorption change could reflect a redox reaction within the water-splitting enzyme system Y. In this case, Y-320 should be identified as S_3 which disappears with $\tau_{1/2} = 1–2$ ms by oxidation. This assumption indicates that either the dioxygen-complex does not significantly absorb at 320 nm or that the release of molecular oxygen from system Y takes place much faster than its oxidative formation. For the time being, experimental evidence is lacking to substantiate Y-320. Further investigations are required to clarify this point.

These data additionally show, that mildly trypsinized chloroplasts in the presence of $K_3[Fe(CN)_6]$ provide an appropriate assay for functionally isolated systems II.

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