

Kinetics of the Back Reaction of Photosystem II as Derived from Delayed Light Emission in the Presence of DCMU and the Conformational State of the Thylakoid Membrane

Gerhard Vierke

Institut für Physikalische Biochemie und Biochemie, Universität Frankfurt, Sandhofstr. 2–4,
D-6000 Frankfurt 71

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It is shown that the kinetics of the back reaction of photosystem II in the seconds time range as derived from the luminescence decay curve in the presence of DCMU is controlled by the internal pH of the thylakoids. Modifications of the conformational state of the photosynthetic membrane while leaving the internal pH unchanged, however, may strongly affect the kinetics of the back reaction.

Introduction

According to the present view of the primary reactions in photosynthesis illumination of the photosynthetic membrane results in the separation of charges across the membrane that can be reversed in the dark by a back reaction. Though conclusive evidence is still missing, some experiments strongly argue in favor of Q^- and the S states being the ultimate electron donor or acceptor, respectively, of this reaction [1–3]. According to the recombination hypothesis of luminescence [3] delayed light emission is thought to be associated with the back reaction. Therefore, the kinetics of the back reaction may be measured by following the luminescence decay curve [4–6]. This method is especially useful in cases where the deactivation of the S states upon reduction through the back reaction cannot be investigated directly by oxygen evolution measurements *e. g.* after inhibiting oxygen evolution by electron transport inhibitors.

The investigation of the kinetics of the deactivation process which may contribute to the elucidation of the real nature of the S states is complicated, however, by the fact that the back reaction is not a localized process within the photosynthetic membrane but involves electron back transfer across the membrane. Hence it is expected to be affected by several macroscopic factors such as membrane potential and the conformational state of the membrane.

It has been shown [7–14] that delayed light emission is influenced by the high-energy state of photophosphorylation. It is thought that the proton motive force – the combined force of the transmembrane electric field and of the electrochemical gradient of the proton across the membrane – reduces the activation energy barrier for delayed light emission [9–11]. Though there is experimental evidence supporting this notion (for review see *e. g.* [15]), the recent findings of Jursinic *et al.* [16] have thrown some doubt on the general validity of this concept. It was observed that millisecond delayed light after a single excitation flash is enhanced *only* if a proton gradient is present. The light induced potential generated across the membrane, however, was found to be 130 mV. It was hypothesized that the internal pH might be the rate controlling factor for delayed light emission rather than the pH gradient. Since the internal pH is thought to be the predominant factor regulating membrane ultrastructure [15, 17] the kinetics of the back reaction and of delayed light emission associated with it would then be expected to depend strongly on the conformational state of the thylakoid membrane. In this paper this is shown to be true.

Materials and Methods

Preparation of the Chlorella samples

Chlorella fusca was cultivated as described by Soeder *et al.* [18]. *Chlorella* cells were taken from a synchronous culture always at the same time shortly after the release of the autospores (in the 24th h of

Reprint requests to Dr. Gerhard Vierke.

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the synchronous cycle). They were kept in dark until use within one h later. The cells were then harvested by centrifugation at 25 °C, washed and resuspended in 67 mM potassium phosphate at pH values and Chl concentration as indicated in the legends to figures. After addition of 20 µM DCMU the suspension was continuously stirred for 10 min.

DCMU was obtained from K & K Lab. and was recrystallized twice from benzene.

Chlorophyll was determined as described previously [6].

NH₂OH extraction of *Chlorella* was carried out according to Cheniae and Martin [19, 20] as described previously [5].

Preparation of spinach chloroplasts

Chloroplasts were isolated from market spinach according to the following procedure. Approximately 20 g of fresh spinach leaves (without ribs) were suspended in a buffer solution containing 50 mM TES-buffer pH 7.9, 0.4 M sucrose, 10 mM NaCl, 20 mM ascorbate, and 5 mM MgCl₂. They were homogenized for 10 sec in a blender, filtered through two layers of nylon cloth (mesh width 70 × 70 µm), and centrifuged for 5 min at 200×g. The supernatant was then centrifuged at 1000×g for 15 min. The sediment was incubated in the isolation buffer and was stored at 0 °C until use one h later. Chlorophyll was determined by the method of Arnon [21]. The chloroplasts were incubated with DCMU for 10 min in the dark.

Luminescence measurements

Luminescence was excited by monochromatic light (478 nm ± 10 nm) obtained from a 900 W Xenon lamp (XBO 900 W, Osram, placed in the LH 151 NZ lamp house, Schoeffel Instr.). The exciting light beam was passed through a water filter (10 cm), a IR reflection filter and through a monochromator (Bausch & Lomb 33-86-02 with grating 33-86-25-02, blaze 500 nm). The monochromatic light was focussed on the cuvette (made of quartz glass suprasil, Hellma) containing the *Chlorella* suspension. The intensity of the exciting light measured at the surface of the cuvette was 22 mW/cm². The optical path-length of the suspension was 5 mm. Chlorophyll concentration was usually kept well below 50 µg/ml Chl_{tot} in order to minimize reabsorption of delayed light.

The temperature of the cuvette was regulated by a thermostat and was measured by a calibrated copper-constantan thermocouple. Luminescence was measured in the direction of the excitation beam. The sample was placed in the center of a cylindrical shutter with two openings arranged at an angle of 85 °C so that the sample was either illuminated with exciting light and the emission window was closed or after rotating the shutter by an electrical pulse within 10 ms luminescence was measured with the excitation window closed. The time resolution of the spectrometer, therefore, is 10 ms.

The emitted light was measured by an EMI photomultiplier 9658 A which was kept at -30 °C by use of a thermoelectrically refrigerated photomultiplier tube housing (TE 104, products for Res., Inc. USA) in order to improve the signal-to-noise ratio. The photomultiplier was protected from stray light by a cut-off filter (WG 655, 10 mm, Schott) permitting the measurement of the whole spectrum of luminescence. The photomultiplier signal was fed to a rapid DC amplifier (GV 9031, EGB) and then was recorded by a light beam galvanometer recorder (Lumiscrypt-150-13, Hartmann & Braun). Millisecond flash-induced luminescence was measured by placing an electronic shutter (Compur electronic 5 FS) in the excitation beam. The shortest flash duration available was 16 ms. The electromagnetically driven shutter of the luminescence spectrometer was triggered by an electric pulse from a phototransistor placed at the opening of the electronic beam shutter with the help of a special electronic device. This device also allowed for the selection of various delay times between the incoming pulse of the phototransistor and the trigger pulse for the electromagnet of the spectrometer shutter. Delay times were variable between 0 and 140 ms. So, by using delay times shorter than the duration of the electronic beam shutter, even a one millisecond flash could be generated.

Before each measurement the sample was kept in the dark for 15 min.

Determination of the kinetics of the back reaction

The kinetics of the back reaction in *Chlorella* in the presence of DCMU was determined from the luminescence decay curve according to the method described earlier [5, 6]. This method correlates the partial and total light sums of luminescence with the

time course of the oxidation of the reduced primary electron acceptor Q^- of photosystem II in the seconds region. The theory [5, 6] leads to the following expression

$$\frac{[Q^-]}{[Q^-]_0} = \frac{[1 + 2ABN(t)]^{1/2} - 1}{A}$$

which is valid in *Chlorella* for times $t \geq 0.3$ sec. A and B are constants depending on the values of p , the mean probability for excitation transfer between different photosystem II centers, and of the ratio φ_∞/φ_0 of the fluorescence yields when $[Q^-] = [Q^-]_0$ or $[Q^-] = 0$, respectively. For *Chlorella* p is equal to 0.45 and φ_∞/φ_0 to 5 [5, 6]. In chloroplasts the same values can be used when Mg^{2+} is present [4, 22]. In the absence of Mg^{2+} $\varphi_\infty/\varphi_0 = 2.5$ [22] but p remains unchanged as indicated by the data of Melis and Homann [22]. In NH_2OH extracted *Chlorella* p was shown to be 0.49 and $\varphi_\infty/\varphi_0 = 5$ [5].

$$N(t) = 1 - \frac{\mathcal{L}(t)}{\mathcal{L}_{tot}}$$

$\mathcal{L}(t)$ and \mathcal{L}_{tot} are the partial or total light sums, respectively. The light sums were calculated by integrating numerically the luminescence decay curve. Integration was done by making use of the integration program of the Hewlett Packard calculator 9815 A.

Results and Discussion

It is well known that the photosynthetic membrane changes its ultrastructure upon several treatments *e. g.* upon illumination [15, 24–26], after acidification in the dark [15], and in the presence of Mg^{2+} [15, 26]. Furthermore, it is expected that the structure of the membrane is also changed upon NH_2OH treatment because of the loss of membrane bound Mn. Therefore, we investigated the kinetics of the back reaction in the presence of DCMU in the following conformational states:

1. The stationary light-adapted state. In the presence of DCMU this state is obtained after 30 sec of continuous illumination. Measurement of the induction kinetics of luminescence has shown that the pH gradient is fully developed.

2. In the flash-induced state at pH 8. This state is created by flash-illumination of dark adapted photosynthetic systems incubated in buffer at pH 8. In order not to induce a noticeable pH gradient the

duration of the flash should be sufficiently short (< 20 ms). (It was found, however, that the kinetics of the back reaction did not depend on flash duration in the time range 1–140 ms.) Then only one proton per photosystem is transferred across the membrane and the internal pH is left unchanged. Therefore, the ultrastructure of the membrane in this state is similar to the dark adapted state.

3. In the uncoupled light-adapted state. This state was created by continuous illumination in the presence of an uncoupler.

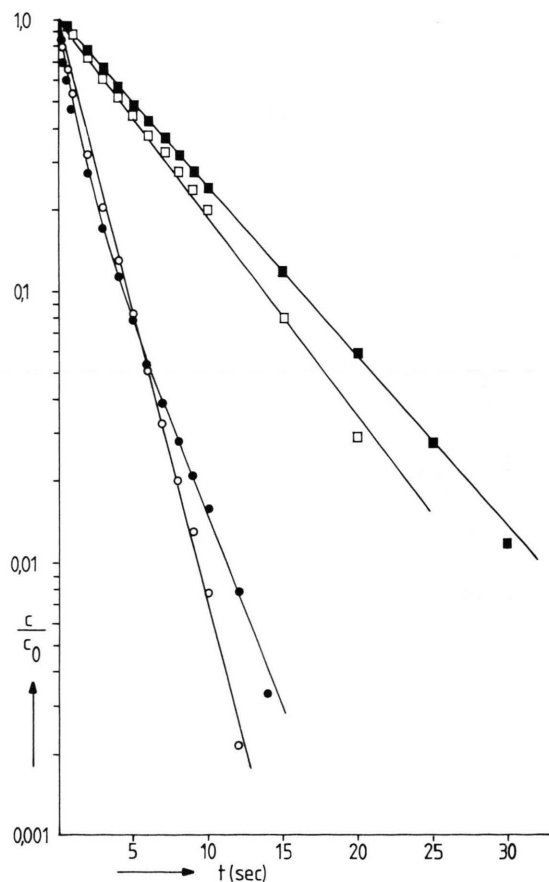


Fig. 1. Kinetics of the back reaction in the flash-induced and stationary light-induced state of the thylakoid membrane in *Chlorella* and in isolated chloroplasts of spinach in the presence of DCMU. \circ Flash-induced state in *Chlorella*; \bullet light-adapted state in *Chlorella*; \square flash-induced state in chloroplasts; \blacksquare light-adapted state in chloroplasts. Temperature, 25 °C. DCMU, 20 μ M. Preillumination times, 15 ms (flash-induced state) and 30 sec (light-adapted state). Chlorophyll concentration, 33.5 mg/l (*Chlorella*) and 50 mg/l (Chloroplasts). *Chlorella* cells were incubated in phosphate buffer at pH 8.0 and chloroplasts in TES-buffer at pH 7.9, containing 0.4 M saccharose, 5 mM $MgCl_2$, 10 mM NaCl and 20 mM ascorbate.

4. In the flash-induced state at pH 5. This state is generated by illuminating the sample which was incubated in a buffer solution at pH 5 in the dark by a short flash.

5. In the state obtained after extraction of membrane bound Mn by NH_2OH treatment. It was observed that release of membrane bound Mn upon NH_2OH treatment leads to structural modifications of the electron acceptor of the back reaction [27, 28]. Therefore, it seems highly likely that loss of membrane bound Mn causes alterations in membrane ultrastructure.

It is seen that the kinetics of the back reaction is different in the flash-induced state and in the stationary light-adapted state of the membrane in *Chlorella* as well as in isolated chloroplasts (Fig. 1). In *Chlorella* the kinetics is first order after flash excitation but not after continuous illumination. In chloroplasts first order kinetics was observed in both states in most of our preparations*.

If it is true that the conformational state of the membrane predominantly depends on the internal pH, the kinetics of the back reaction in the flash-induced state ($\Delta\text{pH } 0$) should be very similar to that observed in the light-adapted state in the presence of an uncoupler. This was found to be true in chloroplasts uncoupled by a high concentration of Valinomycin (Fig. 2) and in *Chlorella* in the presence of 20 mM NH_4Cl (Fig. 2)**.

However, because of the possible dependence of the kinetics of the back reaction on the free energy of

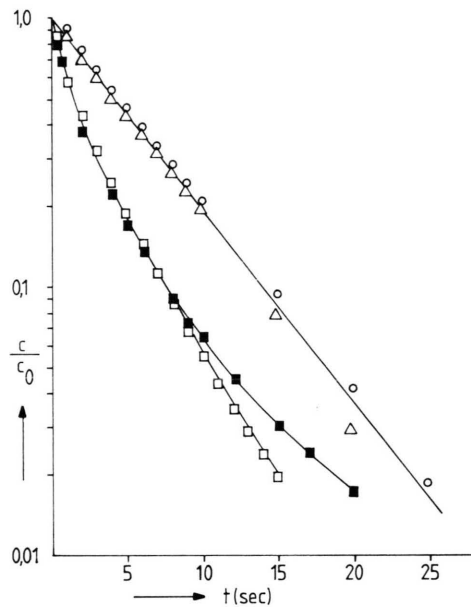


Fig. 2. Effect of uncouplers on the kinetics of the back reaction in the flash-induced and in the light-adapted state of the thylakoid membrane in *Chlorella* and in isolated chloroplasts of spinach in the presence of DCMU. ○ Light-adapted state of the thylakoid membrane in chloroplasts in the presence of 2 μM Valinomycin and 5 mM KCl. Δ Flash-induced state in chloroplasts in the absence of the uncoupler. □ Flash-induced state of the thylakoid membrane in *Chlorella* in the presence of 20 mM NH_4Cl . ■ Light-adapted state in *Chlorella* in the presence of 20 mM NH_4Cl . Temperature, 25 °C. DCMU, 20 μM . Preillumination times, 15 ms (flash-induced state) and 30 sec (light-adapted state). Chlorophyll concentration, 35.5 mg/l (*Chlorella*) and 50 mg/l (chloroplasts). *Chlorella* cells were incubated in phosphate buffer at pH 8.0, and chloroplasts in TES-buffer at pH 7.9, containing 0.4 M saccharose, 5 mM MgCl_2 , 10 mM NaCl and 20 mM ascorbate.

* In some cultures of *Chlorella* slight deviations from first order kinetics were found in the flash-induced state. In chloroplasts deviations were observed occasionally in the stationary light-adapted state but not in the flash-induced state.

** This experiment differs from the Valinomycin experiment in that NH_4Cl was also present in the flash-induced state, though not necessary in order to suppress the pH gradient. This was done for the sake of comparison because NH_4Cl , besides its uncoupling action, exerts an additional effect on the kinetics of the back reaction. It successively slows down the kinetics with increasing NH_4Cl concentration in any state of the membrane.

In the presence of uncouplers which are known to interact with the S states such as CCCP (5 μM) and FCCP (1 μM) luminescence is greatly decreased and the kinetics still being first order is strongly accelerated in chloroplasts. It is different in the two states (enhancement of the rate by the factor 10 in the flash-induced state and by 3.1 in the light-adapted state in the presence of 5 μM CCCP and by the factor 6.9 and 2.7, respectively, in the presence of 1 μM FCCP).

photophosphorylation this result could be due to the absence of the pH gradient rather than to equal values of the internal pH in both states. A decision between these two possibilities can be reached by the following experiment. A low interior pH value may be created not only by long time illumination but also by sufficiently lowering the pH value of the buffer solution in which the algal cells are suspended. Then no significant pH gradient is present and when flash illumination is used no pH gradient will be created. Under these conditions it was found that the same kinetics of the back reaction results in the light-adapted state at pH 8 and in the flash-induced state at pH 5 (Fig. 3). This experiment clearly demonstrates that the kinetics of delayed light emission and consequently, according to the recombina-

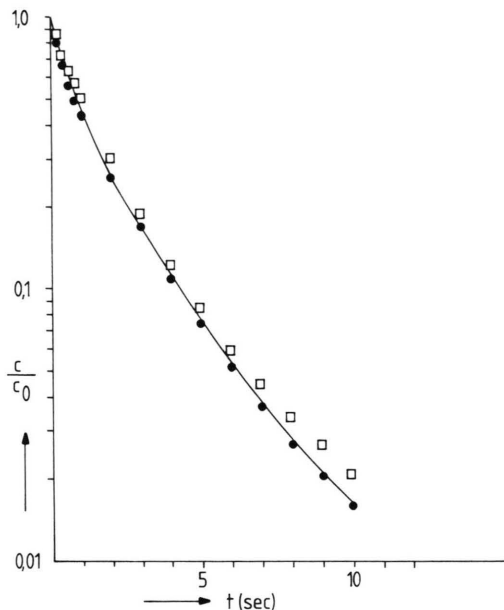


Fig. 3. Kinetics of the back reaction in *Chlorella* in the presence of DCMU in the light-adapted state of the thylakoid membrane at pH 8.0 and in the flash-induced state at pH 5.1. ● Light-adapted state at pH 8.0; □ flash-induced state at pH 5.1. Phosphate buffer with pH values as indicated, Chlorophyll concentration, 34.5 mg/l. DCMU, 20 μ M. Temperature, 25 °C. Preillumination times, 15 ms (□) or 30 sec (●).

tion hypothesis of luminescence, the kinetics of the back reaction is controlled by the internal pH and not by the pH gradient. This view has already been discussed earlier as an alternative hypothesis to the theory of Crofts *et al.* [10] by Jursinic *et al.* [16] but had not been substantiated by appropriate experiments as yet.

The internal pH, however, appears not to be the only rate controlling factor of the back reaction. Changing the conformational state of the membrane while leaving the internal pH unchanged strongly affects the kinetics of the back reaction. This was demonstrated by altering membrane ultrastructure in chloroplasts by MgCl_2 addition and in *Chlorella* by NH_2OH treatment.

The kinetics of the back reaction in chloroplasts is accelerated upon addition of 5 mM MgCl_2 in the light-adapted state but is left unchanged in the flash-induced state of the membrane (Fig. 4). The difference of the kinetics in the two conformational states is greatly diminished but is still significant.

The absence of any effect of Mg^{2+} on the kinetics of the back reaction in the flash-induced state indicates that Mg^{2+} affects the conformational state of the membrane only at low internal pH. This may be due to exchange effects of Mg^{2+} and H^+ ions. The Mg^{2+} -effect on the kinetics of the back reaction is completely developed in the presence of 5 mM MgCl_2 . Addition of higher amounts of MgCl_2 (up to 25 mM) does not give rise to further changes of the kinetics.

The effect of NH_2OH extraction in *Chlorella* was found to be much less pronounced in the light-adapted state (low internal pH) than in the flash-

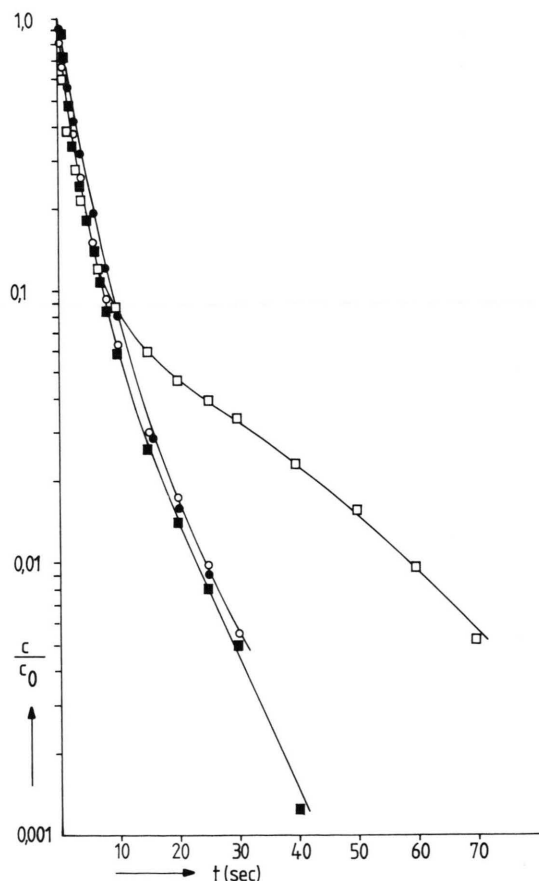


Fig. 4. Kinetics of the back reaction in chloroplasts in the presence and absence of MgCl_2 . □ Kinetics in the light-adapted state in the absence of MgCl_2 (preillumination time 30 sec); ○ kinetics in the flash-induced state in the absence of MgCl_2 (preillumination time 15 ms); ■ kinetics in the light-adapted state in the presence of 5 mM MgCl_2 ; ● kinetics in the flash-induced state in the presence of 5 mM MgCl_2 . 50 mM TES-buffer, pH 7.0, containing 0.4 M saccharose and 10 mM NaCl. Chlorophyll concentration, 50 mg/l. DCMU, 20 μ M. Temperature, 25 °C.

induced state (high internal pH) (Fig. 5)*. In the flash-induced state of the membrane the kinetics is greatly accelerated in NH_2OH treated *Chlorella* as compared to untreated *Chlorella* (Fig. 5). But in the light-adapted state the situation is reversed. The kinetics in NH_2OH treated *Chlorella* is somewhat slower (Fig. 5). These results indicate that the structural changes of the thylakoid membrane upon loss of membrane bound Mn are by far less prominent in the light-adapted state than in the flash-induced state**.

These results suggest that several factors (e. g. internal pH, conformational state) might control the rate of the back reaction.

In the absence of other factors affecting the conformational state of the thylakoid membrane it is not clear, however, whether the internal pH or the conformational state is the primary rate controlling factor. It could well be the internal pH comes into play only indirectly in that it regulates membrane ultrastructure which itself exclusively determines the rate of the back reaction by fixing its activation barrier to a level that corresponds to this specific conformational state.

This conformation hypothesis would be able to explain the findings of Jursinic *et al.* [16] that no enhancement of millisecond delayed light was observed after a single excitation flash in spite of a membrane potential being present. According to this hypothesis the activation energy for the back reaction would be solely determined by the conformational state of the membrane induced by flash-illumination but would not be affected seriously by

* It is well known that NH_2OH treatment impairs only photosystem II activity but not that of photosystem I (see e. g. [28]). Therefore, acidification of the interior space of the thylakoids through the cycling of photosystem I is still observed.

** At this point the objection could be made that alterations of the kinetics of the back reaction observed after NH_2OH treatment could be due to the replacement of the electron acceptor Z^+ by another acceptor D^+ because of the action of NH_2OH on the oxidizing side of photosystem II. However, it is by no means certain whether NH_2OH treatment leads to the destruction of the secondary donor of photosystem II and its replacement by an auxiliary donor D as claimed by P. Joliot [30]. The experiments of Den Haan *et al.* [28] and of A. Joliot [27] do not allow for this conclusion. They merely demonstrate that the secondary donor is modified upon NH_2OH treatment. This modification, however, may simply reflect the change in membrane ultrastructure caused by release of membrane bound Mn by NH_2OH .

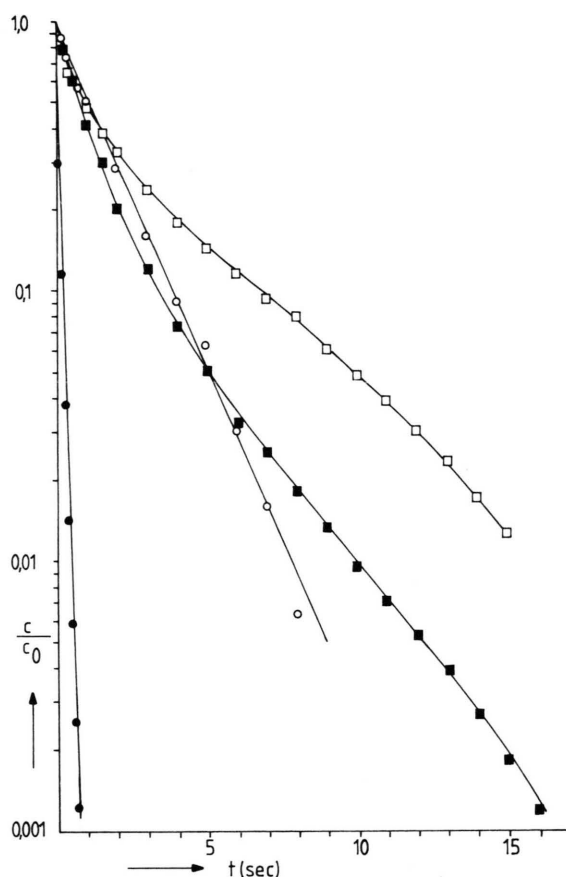


Fig. 5. Kinetics of the back reaction in untreated and NH_2OH treated *Chlorella* in the light-adapted and flash-induced state of the thylakoid membrane in the presence of DCMU. ○ Untreated *Chlorella* in the flash-induced state; ● NH_2OH treated *Chlorella* (20 mM NH_2OH for 20 min) in the flash-induced state; ■ untreated *Chlorella* in the light-adapted state; □ NH_2OH extracted *Chlorella* in the light-adapted state. Temperature, 25 °C. DCMU, 20 μM . Pre-illumination times, 15 ms (flash-induced state) and 30 sec (light-adapted state). Chlorophyll concentration, 34 mg/l, except for □ (55 mg/l). *Chlorella* cells were incubated in phosphate buffer at pH 6.75 (experiments carried out in the light-adapted state) or at pH 7.5 (measurements in the flash-induced state).

the presence or absence of a membrane potential. The enhancement of delayed light observed at low internal pH would be attributed to the lowering of the activation energy induced by the conformational change of the membrane.

The experiments of Barber and Kraan [8] showing that ionic treatments with salts or by pH shifts can induce luminescence from preilluminated chloroplasts have also been interpreted according to the conformation hypothesis [15].

The results presented in this paper, though not providing conclusive evidence for the conformation hypothesis, clearly show, however, that simplistic interpretations of delayed light effects which ignore the influence of the conformational state of the thylakoid membrane on the back reaction of photosystem II may be strongly misleading.

Acknowledgements

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