The Chlorophyll a_{II} Reaction in Trypsin-Treated Spinach Chloroplasts in the Presence of Potassium Ferricyanide

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In trypsin-treated spinach chloroplasts there is no linear electron flow from water to potassium ferricyanide. The chlorophyll a_{II} reaction, however, is still active but insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea. From this we conclude that ferricyanide and trypsin together stimulate a mini-cycle in photosystem II.

Introduction

From measurements of DCIP-reduction Mantai [1] has shown that trypsin-treatment of chloroplasts leads to an initial increase of the electron flow (uncoupling) followed by an inhibition of electron transport from water to DCIP. From measurements of $NADP^+$-reduction in the presence of the photosystem I donor system, DCIP plus sodium ascorbate, the same author concluded that the reaction site of trypsin is not located within photosystem I [2]. From measurements of DCIP-reduction in the absence and in the presence of a photosystem II donor system and from fluorescence yield measurements Selman and Bannister [3] concluded that trypsin inhibits electron transport at least two sites on the oxygen producing side of photosystem II. The conclusions of these authors have been confirmed by measurements of the absorbance changes of chlorophyll a_I and chlorophyll a_{II} in the absence and in the presence of donor systems of photosystem I and photosystem II and by measurements of the fluorescence induction and of the absorbance changes of the electrochromic effect [4]. From measurements of the DCIP-reduction in Tris-treated chloroplast membranes from *Chlamydomonas reinhardi* in the presence of DPC as electron donor Regnitz and Ohad [5] concluded that trypsin-treatment leads to a destruction of the water splitting system and not to an inactivation of the active center of photosystem II. In such chloroplast membranes (Tris-treated and trypsin-treated) the electron transport from DPC to DCIP is not sensitive to DCMU [5].

In this paper further investigations of the photoreactions of chlorophyll a_I and chlorophyll a_{II} in trypsin-treated spinach chloroplasts are reported. Gläser *et al.* [6] found that the kinetics of the chlorophyll a_{II} reaction is biphasic. Besides the well known 200 μs absorbance changes [7] they observed a fast component with a half-life time of approx. 35 μs. In this paper only the 200 μs component of the chlorophyll a_{II} reaction has been investigated.

Materials and Methods

Stripped spinach chloroplasts were isolated as described in [8]. Trypsin-treatment was performed as follows. Trypsin was added to 1 ml of chloroplast suspension containing 100 μM chlorophyll, 500 μM potassium ferricyanide, 2 mM NH_4Cl and 20 mM Tris-HCl-buffer pH 7.2. This suspension was held in the dark for 10 min. Further additions, when used, were made after the 10 min darktime just before the start of measurements. Measurements took about 4 min.

The measurements of the absorbance changes were performed by the repetitive flash technique described in [7]. Excitation: 385 – 500 nm (2 mm BG 28 + 2 mm KG 2 from Schott) for measurements between 600 and 710 nm, 610 – 710 nm (4 mm RG 610 + 2 mm KG 2 from Schott) for measurements between 400 and 580 nm, saturating flashes of 20 μsec duration, repetition rate 10 Hz. The electrical band width ranged from 0.1 Hz to 37 kHz. The optical path length through the cuvette was 1.2 mm. The band width of the monitoring light (grating monochromator) was 5 nm, the intensity about 50 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}. The temperature of the sample was 22 °C.

Abbreviatinos: DCIP, 2,6-dichlorophenolindiphenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, N-methylphenazonium sulphate; DPC, 1,5-diphenylcarbacide.

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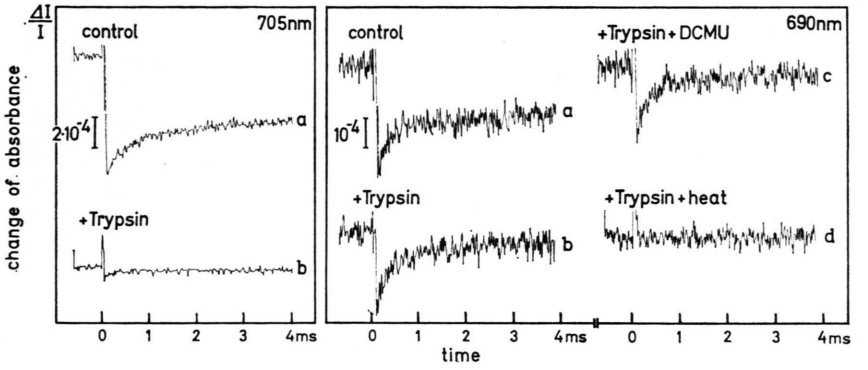

Fig. 1. Left: Changes of absorbance at 705 nm in spinach chloroplasts as a function of time. a. untreated chloroplasts, b. trypsin-incubated chloroplasts. Right: Changes of absorbance at 690 nm in spinach chloroplasts as a function of time. a. untreated chloroplasts, b. trypsin-incubated chloroplasts, c. trypsin-incubated chloroplasts plus 4 μM DCMU, d. trypsin-incubated chloroplasts after a 5 min heat-treatment at 60 °C.

Results

The absorbance changes of chlorophyll a_I and chlorophyll a_{II} (at 705 nm and 690 nm resp.) were investigated in trypsin-treated spinach chloroplasts. When the electron acceptor is potassium ferricyanide (FeCy), the 10 min trypsin-treatment (80 μg/ml) leads to the following results (see Fig. 1). At 705 nm the absorbance changes of chlorophyll a_I are inhibited nearly completely; the residual signal height is less than 10% compared to the control. At 690 nm the slow component (chlorophyll a_I) is inhibited, but the fast component (chlorophyll a_{II}) is completely uneffected. The chlorophyll a_{II} absorbance changes in trypsin-treated chloroplasts are insensitive to DCMU; it does not matter whether DCMU is added before or after trypsin-treatment. Heat-treatment (5 min at 60 °C) leads to a complete inhibition of the absorbance changes at 690 nm. In the absence of an electron acceptor there are no absorbance changes of as well chlorophyll a_I as chlorophyll a_{II} in trypsin-treated chloroplasts (not shown in Fig. 1).

The extent of the slow (chlorophyll a_I) and the fast component (chlorophyll a_{II}) of the absorbance changes at 690 nm as a function of the trypsin-concentration is shown in Fig. 2. The slow component shows a rapid decrease with increasing trypsin-concentration down to less than 20% of the control. The concentration of half inactivation is less than 1.6 μg trypsin/ml. These findings are in agreement with the results obtained with benzyl viologen as electron acceptor (see l. c. 4). However, in contrast to the slow component the fast component is independent on the trypsin-concentration up to at least 160 μg/ml, and also the half-life time of the fast component (approx. 160 μs) does not depend on the trypsin-concentration.

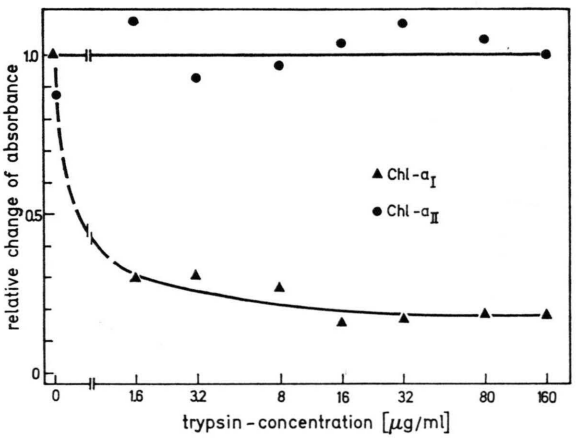

Fig. 2. Absorbance changes at 690 nm with a life time of approx. 160 μs (●) and approx. 10 ms (▲) in spinach chloroplasts as a function of the trypsin-concentration.

The extent of the slow (chlorophyll a_I) and the fast component (chlorophyll a_{II}) of the absorbance changes at 690 nm as a function of the trypsin-concentration in the presence of 4 μM DCMU is shown in Fig. 3. The slow component shows a slight increase with increasing trypsin-concentration, but the extent is always less than 15% of the control. For trypsin-concentrations higher than 1.6 μg/ml the fast component increases with increasing trypsin-concentration up to the extent in untreated chloroplasts. The concentration of half reactivation is approx. 6 μg/ml.

The difference spectrum of the fast component ($\tau_{1/2} \approx 160$ μs) in trypsin-incubated chloroplasts in the presence of 4 μM DCMU is shown in Fig. 4. The maximum in the red region (685 nm) is in the same range of wavelength as the red maximum of the difference spectrum of chlorophyll a_{II} [9–11]. At wavelengths between 415 and 650 nm no significant changes with a half-life of approx. 160 μs occured.

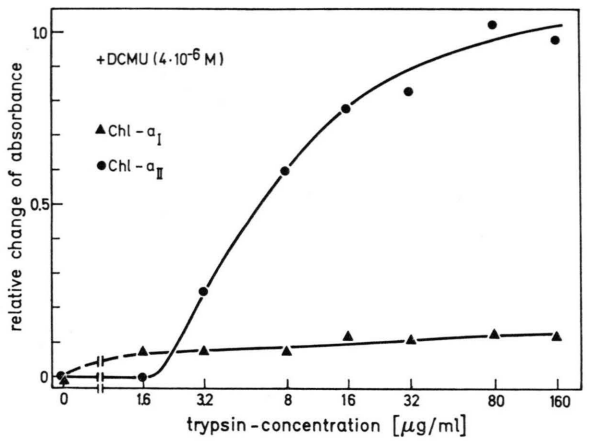

Fig. 3. Absorbance changes at 690 nm with a life time of approx. 160 μs (●) and approx. 10 ms (▲) in spinach chloroplasts as a function of the trypsin-concentration in the presence of 4 μM DCMU.

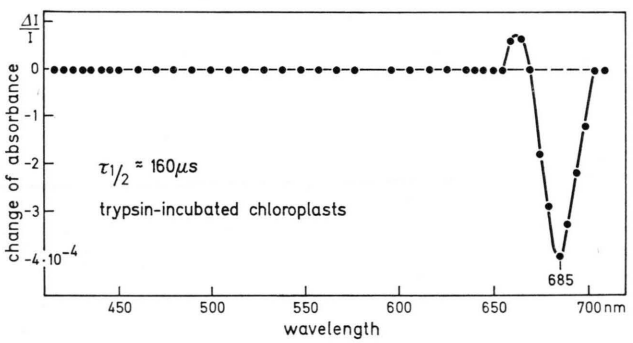

Fig. 4. Absorbance changes with a life time of approx. 160 μs as a function of the wavelength in trypsin-incubated spinach chloroplasts in the presence of 4 μM DCMU.

In agreement with Regnitz and Ohad [5] we found no reduction of potassium ferricyanide in trypsin-treated chloroplasts.

Discussion

We have shown that trypsin-treatment of chloroplasts leads to an inhibition of the photoreactions of chlorophyll a_I and chlorophyll a_{II}, when the electron acceptor is benzyl viologen, and that the chlorophyll a_I reaction can be reactivated by reduced PMS, whereas a reactivation of the chlorophyll a_{II} reaction by reduced HQ (*p*-benzo-hydroquinone) is not possible [4].

The results reported in this paper have shown that trypsin-treatment leads to an inhibition of the chlorophyll a_I reaction as well when ferricyanide is used as electron acceptor instead of benzyl viologen or in the absence of an electron acceptor. The chlorophyll a_{II} reaction, however, in contrast to the results with benzyl viologen and without an electron acceptor is not inhibited after trypsin-treatment, when the electron acceptor is ferricyanide. Under these conditions the chlorophyll a_{II} reaction is not sensitive to DCMU. With decreasing trypsin-concentration the inhibitory effect of DCMU to the chlorophyll a_{II} reaction increases (see Fig. 3). This unsensitiveness of the active center of photosystem II to DCMU in trypsin-treated chloroplasts is in agreement with the results reported by Regnitz and Ohad [5].

Because the Soret band is missing in the spectrum of the absorbance changes with a half-life of approx. 160 μs (see Fig. 4), one could assume that the absorbance changes around 685 nm do not belong to chlorophyll a_{II}. However, of the cell pigments in the chloroplasts only the chlorophylls of the type "a" absorb in this long wavelength region, and the half-life as well as the extent of the fast component of the absorbance changes at 690 nm do not change with increasing trypsin-concentration (see Fig. 2). Because of this constancy of the half-life and the extent of the absorbance changes it is very probable that the absorbance changes in trypsin-treated chloroplasts belong to the same component as those in untreated chloroplasts.

Assuming that the absorbance changes in the region around 685 nm belong to chlorophyll a_{II}, there arise two questions: 1. Why is the chlorophyll a_{II} reaction insensitive to DCMU under these conditions, and 2. why don't we find the Soret band of chlorophyll a_{II} under these conditions? We can interpret our experimental results as follows.

Selman and Bannister [3] have shown that one site of inhibition by trypsin is located between the site of inhibition by Tris and photosystem II. If this reaction site of trypsin is located between Y_2 and Y_1 (see Fig. 5 of ref. 11), the cyclic electron flow in photosystem II, which was found in Tris-washed, in heat-treated, in aged [10] and in deoxycholate-treated chloroplasts [11], cannot operate. However, possibly in trypsin-treated chloroplasts in the presence of ferricyanide the linear electron flow in photosystem II is replaced by another cyclic one, which does not include Y_2 and which is not sensitive to DCMU. This cyclic electron flow does not operate in the presence of trypsin or ferricyanide alone, but only in the presence of both trypsin and ferricyanide.

The absence of the Soret band in the chlorophyll a_{II} difference spectrum (see Fig. 4) in trypsin-treated chloroplasts is not easy to understand. One possible explanation of this behavior would be the assumption that trypsin-treatment alters the surroundings of the chlorophyll a_{II} molecules in such way that the photoreaction of chlorophyll a_{II} in contrast to untreated chloroplasts requires the excited single state S_2 (2.8 eV, corresponding to the absorbance changes at 435 nm) instead of S_1 (1.8 eV, corresponding to the absorbance changes at 685 nm). Because the S_2 state can be excited only by blue light and not by red light, irradiation of blue flashes leads to a chlorophyll a_{II} reaction, whilst irradiation of red flashes does not. Irradiation of red flashes can excite only the S_1 state, which will be depopulated *via* dissipation processes in a time shorter than detectable with our equipment.

Using this model, we expect absorbance changes of chlorophyll a_{II} with a half-life time of 160 μs under excitation with blue flashes, but not with red flashes. This corresponds to the experimental results shown in Fig. 4. The difference spectrum was obtained with red flashes for wavelengths $\leqq 580$ nm and with blue flashes for wavelengths $\geqq 600$ nm, and we find absorbance changes in the red but not in the blue. One should expect absorbance changes of chlorophyll a_{II} in the blue region too under excitation with blue flashes. However, it is not possible to do this experiment with our equipment, because the electronics is overmodulated totally when monitoring light and exciting light have the same wavelength.

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