TRYPSIN INHIBITION OF CYCLIC PHOTOPHOSPHORYLATION

H. STROTMAN, Renate WENINGER and Christa VON GÖSSELN
Botanisches Institut der Tierärztlichen Hochschule,
3 Hannover, Bünteweg 17, Germany

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1. Introduction

Trypsin treatment of isolated chloroplasts has been shown to inhibit non-cyclic electron flow from water to various Hill oxidants including DPIP [1–3], ferricyanide [4], methylviologen [3], and NADP [1, 4]. On the other hand photosystem I-dependent reduction of methylviologen [3] and NADP [1] by the artificial electron donor system ascorbate plus DPIP is not at all impaired by (short time) trypsin digestion. Therefore trypsin is suggested to attack electron transport close to photosystem II. Selman and Bannister [3] contributed evidence for two different trypsin sensitive sites being located between the water splitting system and photosystem II. Moreover, trypsin affects photosystem I-dependent cyclic phosphorylation catalyzed by methylviologen, but does not inhibit system I-dependent non-cyclic phosphorylation [5].

In this paper the effect of short time trypsin digestion of chloroplasts on PMS mediated cyclic phosphorylation is described. The results indicate two different cyclic systems catalyzed by PMS, depending on the concentration of reduced form of PMS. Only one of the cycles is inhibited by short time trypsin proteolysis of the chloroplast membranes. The inhibition of phosphorylation is shown to be due to an inactivation of electron transport component(s).

Abbreviations:
DCMU : 3,4-dichlorophenyl-1,1-dimethylurea;
DPIP : 2,6-dichlorophenolindophenol;
$DPIPH_2$: reduced DPIP;
PMS : phenazine methosulfate;
tricine : N-Tris(hydroxymethyl)-methylglycine.

2. Methods

Chloroplast preparation from spinach was described elsewhere [6]. Before using the chloroplasts the chlorophyll content of the suspension was adjusted to 1.25 mg/ml according to the method of Arnon [7]. 1 ml of the chloroplast suspension was then incubated at room temp with 1 ml trypsin solution, containing 0.25 mg bovine trypsin (Merck) in 1 ml 50 mM tricine buffer, pH 8. During incubation the mixture was stirred. Trypsin digestion was interrupted by adding 0.5 ml trypsin inhibitor solution, containing 1.5 mg soy bean trypsin inhibitor (Merck) per 1 ml distilled water. For controls trypsin solution was first mixed with the inhibitor and then added to the chloroplasts. After trypsin treatment the samples were put into an ice bath and kept cool until used for the experiments.

The experiments were performed at 20°. The method for measurement of ferricyanide was described elsewhere [6]. Phosphorylation was followed by incorporation of ^{32}P-labelled phosphate into the organic phosphate fraction [6]. The reactions were carried out in small test tubes or Thunberg tubes [5]. In the first case oxygen was removed from the medium by bubbling pure nitrogen through it. During incubation with chloroplasts a vigorous stream of nitrogen was blown over the surface of the reaction mixture. If the reactions were carried out in Thunberg tubes, they were started by illumination after the air had been exchanged 3 times against nitrogen [5]. With both methods corresponding results were obtained.

Measurement of pH changes of the chloroplasts has been described in a previous paper [5].

3. Results

In a previous paper [5] short time trypsin treatment of chloroplasts was shown to inhibit photosystem I-dependent methylviologen mediated cyclic phosphorylation. In contrast non-cyclic phosphorylation coupled to electron flow from $DPIPH_2$ to methylviologen was not affected. These results indicate that trypsin treatment inactivates a component involved in cyclic phosphorylation. The sensitive site is different from that responsible for trypsin inhibition of Hill reaction with various oxidants. Comparing 50% inhibition, Hill reaction is about twice as sensitive toward trypsin as methylviologen mediated cyclic phosphorylation.

Under certain conditions PMS catalyzed cyclic phosphorylation is affected by trypsin quite similar to methylviologen-cyclic phosphorylation. In order to obtain pure system I-dependent cyclic system, electron flow from photosystem II was interrupted by DCMU. In the presence of DCMU the reaction has to be run under anaerobic conditions due to the rapid reaction of oxygen with reduced PMS [8]. In order to obtain maximum phosphorylation rates, PMS has to be added in its reduced form, at least to a certain extent. Reduction of PMS can be achieved by ascorbate.

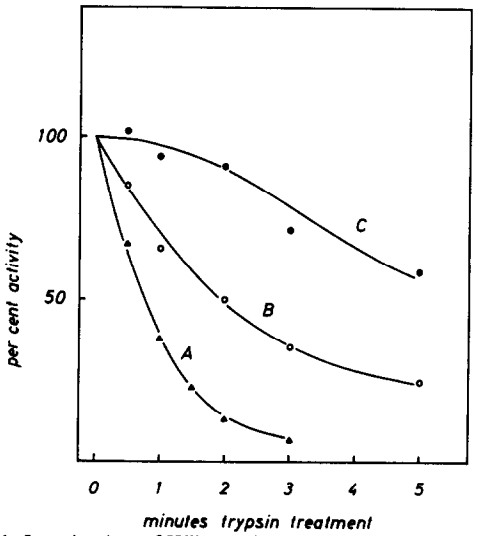

Fig. 1. Inactivation of Hill reaction (A) and PMS-cyclic phosphorylation (B, C) by trypsin treatment of the chloroplasts. The PMS concentration was 30 μM. In B 15 μM ascorbate and in C 300 μM ascorbate were added. The incubation media contained: 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$; in addition 1 mM K_3 [$Fe(CN)_6$] and 10 mM methylamine in A. In B and C 5 mM P_i (^{32}P-labelled), 1 mM ADP, 20 μM DCMU and PMS plus ascorbate were added. The chlorophyll concentration was 25 μg per ml. The samples were illuminated 1 min with 8.5 × 10^5 ergs/cm^2 sec red light (T_{max} = 630 nm) under nitrogen atmosphere (see "Methods"). The control rates were 394 μmoles ferricyanide reduced/mg chlorophyll × hr (A), 561 μmoles ATP/mg chlorophyll × hr (B), and 735 μmoles ATP/mg chlorophyll × hr (C), respectively.

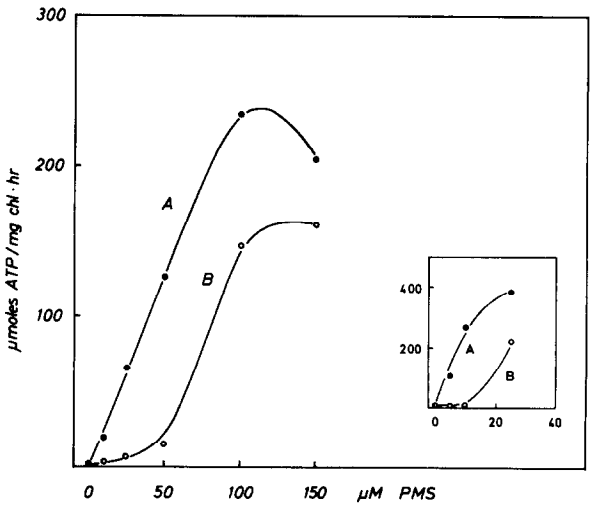

Fig. 2. PMS-cyclic phosphorylation of untreated (A) and 3 min trypsin treated chloroplasts (B) on dependence of PMS concentration. The molar ratio ascorbate/PMS was kept at 0.5 and 10, respectively (inset of the figure), at any PMS concentration. Other experimental conditions see fig. 1.

In the presence of 30 μM PMS and 15 μM ascorbate cyclic phosphorylation is inactivated by trypsin at a rate comparable to inhibition of methylviologen mediated cyclic phosphorylation (fig. 1). Under the conditions used, 50% inhibition is attained after about 2 min of trypsin incubation (curve B). At the same time ferricyanide Hill reaction is nearly completely inactivated (curve A). If the concentration of ascorbate is raised up to 300 μM, a completely different time course of inhibition of cyclic phosphorylation is observed (curve C). During the first 2 min of trypsin treatment the rate of phosphorylation is nearly unchanged. After that time the activity decreases slightly. Trypsin sensitivity of PMS-cyclic phosphorylation at high ascorbate concentration resembles that of non-cyclic system I-dependent phosphorylation [cf. 5].

From the results pointed out in fig. 1 one might speculate that the different sensitivity of PMS-cyclic phosphorylation toward trypsin proteolysis is related to the redox state of PMS. Actually it is due to the total concentration of the reduced form rather than the redox state of the electron mediator. In fig. 2 the effect of different PMS concentrations on the phosphorylation of untreated and trypsin treated chloroplasts is shown. Ascorbate was added to keep a constant ratio ascorbate/PMS of 0.5 at any PMS concentration. At low PMS concentrations a strong trypsin inhibition is found. With increasing PMS concentrations the relative inhibition decreases. In contrast to the control curve the concentration curve of treated chloroplasts appears sigmoid at low PMS concentrations. That means that in trypsin digested chloroplasts a certain PMS concentration has to be exceeded before phosphorylation can take place. At an ascorbate/PMS ratio of 0.5 the critical PMS concentration is about 50 μM. If the ratio is raised up to 10, the critical concentration decreases to about 10 μM (inset of fig. 2). Moreover the saturating PMS concentrations of control chloroplasts equally depends on the ratio ascorbate/PMS. Thus the sensitivity of cyclic phosphorylation toward trypsin as well as the phosphorylation rates of the controls depend on the actual concentration of reduced PMS rather than the redox state of PMS.

The experiments pointed out in figs. 1 and 2 can not give an information on the sensitive reaction responsible for trypsin inhibition of cyclic phosphorylation. Inhibition of phosphorylation may indicate uncoupling or inhibition of energy transfer or inhibition of electron transport as well. One can not distinguish between the possibilities by measuring phosphorylation only.

Light dependent uptake of protons can be taken as a measure of cyclic electron transport. Critical measurements have shown that in non-cyclic systems there is a fixed stoichiometry between proton uptake and electron transport (cf. [9]). Probably this is also consistent in cyclic electron transport systems. Thus proton uptake would reflect cyclic electron flow. Inhibition of cyclic electron transport would decrease the rate of proton uptake, while uncoupling would increase the rate of proton release from the chloroplast thylakoids [10, 11]. The rate constant of proton efflux can be calculated from the half time of the proton gradient upon switching off the light, provided that proton efflux is a first order reaction.

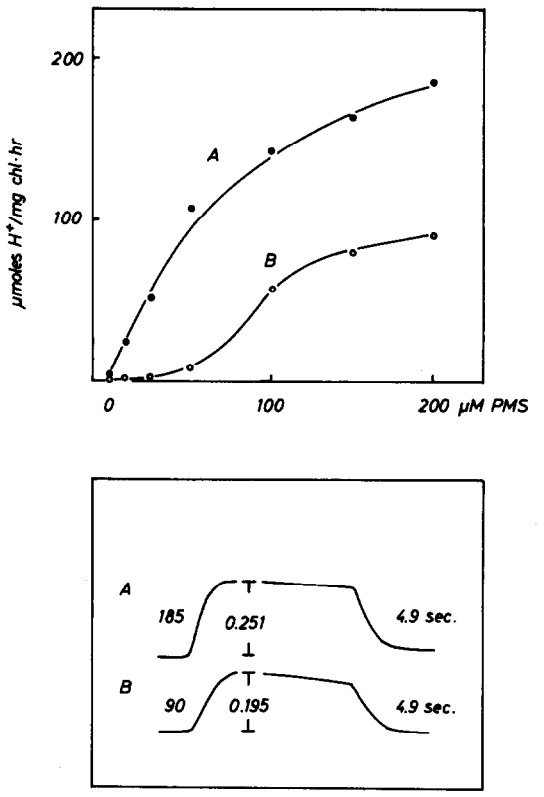

Fig. 3. Initial rate of proton uptake of untreated (A) and 3 min trypsin treated chloroplasts (B) on dependence of PMS concentration. The molar ratios ascorbate/PMS were adjusted to 0.5. In the lower part of the figure original curves of pH changes of control chloroplasts (A) and treated chloroplasts (B) at 200 μM PMS are shown. The figures at the traces indicate the initial rates (in μmoles H^+/mg chlorophyll × hr), the extent of proton gradients (in μmoles H^+/mg chlorophyll), and the half times of the gradients (in sec) upon switching off the light. The reaction medium contained: 100 mM NaCl, 5 mM $MgCl_2$, 20 μM DCMU, PMS and ascorbate, and 38.5 μg/ml chlorophyll. Illumination with 6.7×10^5 ergs/cm^{2}sec red light. The experiments were run under nitrogen atmosphere.

In fig. 3 the effect of PMS concentration on the rate of proton uptake of untreated (curve A) and trypsin treated chloroplasts (curve B) is demonstrated. The experimental conditions resemble those which were applied in the experiments shown in fig. 2. Corresponding to phosphorylation proton uptake is much more inhibited by trypsin at low PMS concentrations than at higher concentrations. On the other

hand the half time of the proton gradient is not affected by 3 min trypsin digestion of the chloroplasts. That means trypsin inhibits cyclic electron flow rather than uncouples phosphorylation from electron transport.

4. Discussion

The main results of the experiments described above are to be summarized as follows:

1) PMS cyclic electron transport is suppressed by trypsin treatment of the chloroplasts when the concentration of reduced form of PMS is low;
2) it is not influenced by the proteolytic enzyme when the concentration of reduced PMS is high.
3) The inhibitory site is different from that which causes inhibition of Hill reaction.

From 1) and 2) we conclude that reduced PMS can enter the electron transport chain at two different sites between the photosystems. To one of the sites electrons are fed from low concentrations of reduced PMS. This cycle (or pseudocycle) is sensitive to trypsin. The second cycle which is induced by high concentrations of reduced electron carrier, is not impaired by the enzyme.

The trypsin sensitive component may be located between the photosystems or in the cyclic pathway induced by low concentrations of reduced PMS. If the alternative is true, the entering site of high PMS concentration would be located behind the trypsin sensitive electron transport component. This cycle would resemble that induced by DPIP (cf. [5]), which is also insensitive to trypsin treatment.

At least in photosystem I-dependent systems uncoupling by trypsin is a relatively slow reaction, although trypsin is known to activate the coupling factor to yield Ca^{2+} dependent-ATPase activity [12]. Under our conditions Ca^{2+}-dependent ATPase is stimulated from 10 to 36 μmoles P_i released/mg chlorophyll X hr during the first 2 min of trypsin treatment. This is a quite low stimulation considering that at maximum activation ATPase yields about 600 μmoles P_i/mg chlorophyll X hr [12].

Acknowledgement

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