Trypsin-sensitive Photosynthetic Activities in Chloroplast Membranes from *Chlamydomonas reinhardi*, y-1*

(Received for publication, August 19, 1975)

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Location of electron transport chain components in chloroplast membranes of *Chlamydomonas reinhardi*, y-1 was investigated by use of proteolytic digestion with soluble or insolubilized trypsin. Digestion of intact membrane vesicles with soluble trypsin inactivates the water-splitting system, the 3-(3,4-dichlorophenyl)-1,1-dimethylurea inhibition site of Photosystem II, the electron transport between the two photosystems as well as the ferredoxin NADP reductase. Reduction of NADP with artificial electron donors for Photosystem I could be restored, however, by addition of purified reductase to trypsin-digested membranes. Electron transfer activities of Photosystems I and II reaction centers were resistant to trypsin digestion either from outside or from within the thylakoids when active trypsin was trapped inside the membrane vesicles by sonication and digestion carried out in the presence of trypsin inhibitor added from outside. In the latter case, the water-splitting system was also found to be resistant to digestion. Polyacrylamide-bound insolubilized trypsin inactivated only the ferredoxin NADP reductase. Phototrophically active membranes obtained at different stages of development showed a basically similar behavior toward trypsin.

The organization of photosynthetic membranes of higher plants has been investigated by many workers using controlled digestion with proteolytic enzymes (1-7). In this way, information was obtained on the localization of several components of the electron transport chain on the outer surface of the thylakoid. Such studies have not yet been extended to chloroplast membranes obtained at different stages of development of either higher plants or unicellular algae such as *Chlamydomonas*. This organism, however, presents several advantages for the study of membrane organization due to the availability of a variety of membrane mutants (8) as well as mutations in which the formation of membrane structure and function can be artificially modulated (9). In addition, digestion of the membrane can be carried out from either side. Soluble trypsin can be introduced within the thylakoid by mild sonication. Also, it is advantageous to use insolubilized trypsin covalently bound to relatively large particles which can reach proteins located only on the outer surface of the thylakoid. In order to use proteolysis as a tool for the study of changes in the chloroplast membrane organization at different stages of its formation, it was first necessary to characterize the effect of controlled proteolysis on the activity of normally functioning membranes. In the present work, the effect of trypsin acting on the outer and inner surfaces of photosynthetic membranes of *Chlamydomonas reinhardi*, y-1 has been studied.

* This work was supported by Grants 184 from the United States-Israel Binational Science Foundation and DR 28/17 from the Deutsche Forschungsgemeinschaft.

† Recipient of postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

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**MATERIALS AND METHODS**

**Isolation of Thylakoid Membranes—*Chlamydomonas reinhardi***, mutant y-1 was grown in the light or dark in a mineral medium supplemented with acetate (10). Greening of dark grown cells was carried out as described before (10). Cells obtained from the logarithmic phase of growth in the light or at different times of a greening experiment were washed by centrifugation and suspended in a solution containing 30 mM Tris, pH 8.0, and 10 mM KC1 (Tris-KC1 buffer) at a final concentration of 10⁴ to 10⁵ cells/ml. In order to break the cells, the suspension was passed through a French press operated at 6000 p.s.i. and 0°C. Large cell debris and unbroken cells were removed by centrifugation at 2000 x g for 2 min. A fraction enriched in chloroplast membranes was sedimented from the supernatant by centrifugation at 27,000 x g for 10 min and resuspended in Tris-KC1 buffer.

**Trypsin Treatment, Sonication, and Trypsin Digestion of Membranes—**Chloroplast membranes were treated with 0.8 mM trypsin buffer, pH 8.3, for 2 min at 0°C according to Yamashita and Butler (11). Membrane suspensions containing 2.5 μg of chlorophyll/ml of Tris-KCl buffer were sonicated for 20 s at 0°C using a Branson sonifier model MS2 equipped with a microtip and operated at 6 A.

Trypsin digestion was carried out at 10°C using 0.5 mg of chlorophyll and the enzyme concentration indicated in the figures in a final volume of 1 ml of Tris-KCl buffer. Proteolysis was stopped by addition of trypsin inhibitor at an inhibitor to enzyme ratio of 4:1 by weight.

**Measurements of Photosynthetic Activities—**The Hill reaction with dichlorophenolindophenol (0.2 mM) or ferricyanide (0.4 mM) and the Mehler reaction with methyl viologen (0.1 mM) in presence of 5 mM sodium azide, 40 μM dichlorophenylindophenol, 5 mM sodium ascorbate, and 0.1 mM dichlorophenolindophenol were measured polarographically at 25°C. Illumination was provided by incandescent light (2.5 x 10³ watts/cm²). The chlorophyll concentration was between 10 and 30 μg/ml of Tris-KCl buffer.

*The abbreviations used are: dichlorophenolindophenol, 2,6-dichlorophenolindophenol; dichlorophenylindophenol, 3-(3,4-dichlorophenyl)-1,1-dimethylyurea; diphenylcarbazide, 1,5-diphenyl-carbazide, Tricine, N-tris(hydroxymethyl)methylglycine.
The photoreduction of dichlorophenolindophenol (0.6 mM) by diphenylcarbazide (1 mM) in Tris-treated membranes (10 μg of chlorophyll/ml) was measured spectrophotometrically in 30 mM phosphate buffer, pH 6.4, using a Aminco-Chance dual wavelength spectrophotometer with the reference beam set at 540 nm and the measuring beam set at 580 nm. Illumination was provided by a 350-watt projector fitted with a Schott RG 665 filter. The photomultiplier was protected by a Corning 4-96 filter.

Photoreduction of NADP was also measured in the Amino-Chance spectrophotometer with the beam set at 340 nm and 390 nm, respectively. Illumination, as above, was provided through Corning 7-51 and Schott OG 515 filters using a Corning 7-37 filter to protect the water to NADP at 10μM. Both reactions were not enhanced by illumination, as above, was provided through Corning 7-51 and Schott OG 515 filters using a Corning 7-37 filter to protect the photomultiplier.

The reaction mixture contained 40 mM Tricine-KOH buffer, pH 8.0; 50 mM KCl; 100 mM sucrose; 0.4 mM NADP; 3.3 μM ferredoxin; excess amounts of ferredoxin NADP reductase when reduced at 25 μg of chlorophyll in a final volume of 3 ml (12). Photoreduction of NADP using artificial electron donors for Photosystem I was measured in presence of 40 μM dichlorophenylindolylmethylene dye, 0.1 mM dichlorophenindolylmethylene dye, and 5 mM sodium ascorbate. In experiments in which electron transfer activity between the two photosystems was measured in Tris-treated membranes, 3 mM diphenylcarbazide was used as electron donor for Photosystem II, and reduction of NADP was followed as described.

Cyclic photophosphorylation was carried out with an open cell preparation as described by Wallach et al. (13). Cytochrome photooxidation was followed according to Schuldiner et al. (14), and chlorophyll content determined after Arnon (15).

Preparation of Samples for Electron Microscopy—For electron microscopy, membranes purified by sedimentation in a linear sucrose gradient (15 to 60%) were used. Membrane samples were collected by centrifugation and the pellet was fixed in glutaraldehyde followed by osmium fixation overnight as described (10). The fixed material was dehydrated and embedded in Epon according to Luft (16). Sections were cut with an LKB Ultratome III microtome, stained with uranyl acetate in 50% ethanol for 1 to 3 min, and photographed with a Philips EM 300 electron microscope.

Reagents—Ferredoxin and plastocyanin were prepared from Chlamydomonas reinhardi, y-1 cell extracts following the procedure of Anderson and McCarty (17). Ferredoxin NADP reductase from Euglena gracilis was kindly given by Dr. E. Tel-Or. Soybean trypsin inhibitor type B, soluble trypsin from bovine pancreas (crystallized twice), and acrylamide-bound trypsin (insolubilized) were purchased in this work were of analytical grade.

RESULTS

Digestion of membranes with trypsin was carried out at 10°C, since it was found that at this temperature, photosynthetic activities are preserved in the nontreated membranes for at least 2 hours. Incubation with trypsin progressively inhibits both photophosphorylation and electron flow under these conditions. Photoreduction of NADP is the most sensitive with 50% inhibition caused by 25 μg of trypsin applied for 30 min, whereas photophosphorylation supported by cyclic electron flow needs 75 μg for the same level of inhibition (Fig. 1).

In order to find out what the specific sites of trypsin action are, partial reactions of the photosynthetic electron transfer chain were tested.

Sensitivity of Photosystem II Activity to Trypsin—Photoreduction for ferricyanide and dichlorophenindolylmethylene dye with water as electron donor were found to be insensitive to 2.5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, which is known to block the electron transport at the level of plastocyanine (18), and completely inhibited electron transport from water to NADP at 10−4 M. Both reactions were not enhanced by light of 680 nm wavelengths and above when carried out with nonsaturative light of 650 nm wavelength.

The photoreduction of ferricyanide was almost completely abolished when membranes were treated for 30 min with soluble trypsin at a concentration of 50 μg/ml, whereas dichlorophenindolylmethylene dye photoreduction was inhibited only to 60% under similar conditions (Fig. 2). These results might indicate that the water-splitting activity was sensitive to trypsin. The fact that trypsin treatment abolished ferricyanide reduction in membranes which were still partially able to photoreduce dichlorophenindolylmethylene dye using the same electron donor system (water) indicated that there exists at least one difference between the ferricyanide and dichlorophenindolylmethylene dye-reducing sites, the former being more sensitive to trypsin.

In order to ascertain this possibility, the reduction of dichlorophenindolylmethylene dye by Tris-washed chloroplast membranes in which the water-splitting system is lost (11) was tested before and after trypsin treatment using diphenylcarbazide as an electron donor. The results show that, indeed, reduction of dichlorophenindolylmethylene dye under these conditions is unaffected by trypsin digestion of the membranes (Fig. 3).

The light-dependent reduction of dichlorophenindolylmethylene dye is sensitive to dichlorophenylindolylmethylene dye and to o-phenanthroline, both specific inhibitors of Photosystem II. However, treatment of the membranes with trypsin abolished the inhibi
Sensitivity of Photosynthetic Electron Transport to Insolubilized Trypsin or Trypsin Trapped within the Thylakoids

Inactivation of photosynthetic electron transport chain by trypsin can be considered as indicative of localization of the membrane components involved on the outer thylakoid surface, if one would be able to show that the membrane preparation used consisted of closed vesicles, and that trypsin does not disrupt them during digestion. Examination of membranes by electron microscopy shows that the majority of the membranes are closed (Fig. 5a) and impermeable to ferritin even after digestion with trypsin (Fig. 5b). Additional information on the localization of trypsin-sensitive membrane components could be obtained by (a) use of insolubilized trypsin which cannot penetrate the thylakoid membrane; (b) digestion of membranes with soluble trypsin trapped within the vesicles by sonication and acting only from within. In this case, trypsin inhibitor should be added after sonication in order to prevent activity of trypsin present in the medium exterior to the thylakoids. As can be seen from Fig. 5c, ferritin which was used as a marker was trapped within the thylakoid following 20 s of sonication which did not cause appreciable loss of photosynthetic activity.

Digestion of the membranes by insolubilized trypsin was found to be effective only against the photoreduction of NADP. The inactivation of NADP photoreduction by insolubilized trypsin could be reversed by addition of excess amounts of purified reductase (Fig. 6).

Digestion of membranes with trypsin trapped within the thylakoids had no effect on any of the photosynthetic electron transport reactions measured (Table 1). The results shown in Table 1 also show that treatment of membranes with trypsin added during sonication without adding trypsin inhibitor, that is, acting from both membrane sides, does result in only a slight additional reduction of the measured activities as compared with that obtained with trypsin acting only from outside. Sonication of the membranes causes a reduction in the activity of Photosystem I reactions, which can be completely restored by addition of purified plastocyanin. This would indicate that sonication causes a partial solubilization of plastocyanin. However, the residual activity of Photosystem I is not decreased following trypsin treatment of the membranes from both sides, and restoration of activity by external plastocyanin is similar in trypsin-treated and nontreated membranes (Table 1). The effects of insolubilized trypsin on the Hill reaction with ferricyanide and of soluble trypsin on photoreduction of methyl viologen were measured at different stages of the greening process of dark grown cells. Preliminary results show that as soon as detectable, these activities have similar sensitivities to digestion as in membranes from light grown cells. Basically similar results have been obtained using chymotrypsin.
FIG. 5. Permeability of thylakoids to ferritin as a function of their sonication or trypsin treatment. a, Ferritin added to nontreated thylakoids. b, Ferritin added to trypsin-digested thylakoids (25 μg/ml, 30 min, 10°), followed by addition of trypsin inhibitor (100 μg/ml). c, Ferritin added during sonication (20 s, 0°) in the presence of trypsin (25 μg/ml), followed by addition of trypsin inhibitor (100 μg/ml). After addition of ferritin, all samples were further incubated for 15 min at 10°, washed by centrifugation, and further processed for electron microscopy. Thin sections were stained briefly with uranyl acetate. Notice that ferritin penetrates the inner thylakoid space only in the sonicated samples. Aggregation of the ferritin in Fig. 5c might be due to the effect of trypsin acting within the thylakoid. Arrows, ferritin attached on the outer surface of the thylakoids seen in tangential sections. × 70,000.

DISCUSSION

The object of this work was to identify the trypsin-sensitive components of electron transport chain in Chlamydomonas reinhardi, y-1 chloroplast membranes and to localize them on the outer or inner surface of the thylakoid. In the interpretation of the data obtained, one can find support in the findings which show that: (a) trypsin acts only on the surface of the membrane unless introduced within the inner space of the thylakoid; the
membranes do not become disrupted or fragmented during digestion as shown by their impermeability to ferritin. The membrane preparation used appears to be free of clumps, aggregation, or stacking, which might prevent trypsin and especially the insolubilized enzyme from coming in contact with membrane surface.

The most likely explanation for the resistance or inactivation of an electron transport activity by proteolysis is that a protein( s) related to this activity is exposed or protected by neighboring membrane components. However, alternative explanations should be considered, e.g., a portion of a protein which is exposed might not have trypsin-sensitive sites, or activity might be preserved after a partial split of the proteins involved. Similar results to those shown in this work have been obtained in our laboratory using also chymotrypsin whose specificity differs from that of trypsin. While several membrane proteins were digested following treatment with either soluble or insolubilized trypsin, proteins which were shown to be associated with the reaction centers of Photosystem I and II (20) were not affected by proteolysis of the membranes. Thus, it seems that the inactivation and resistance of a given function by trypsin are largely associated with the exposure or protection of the proteins involved by the membrane structure.

The results obtained clearly indicate that under our experimental conditions, the activities of both reaction centers of Photosystems I and II are resistant to trypsin acting from either side of the membrane surface. This interpretation is consistent also with results published by Selman et al. (4), who showed that the reaction center of Photosystem I is resistant, and that of Photosystem II only slightly affected by trypsin acting on the outer surface of spinach chloroplast membranes. Cytchrome f photooxidation was found to be resistant to trypsin acting from both sides of the membrane.

However, trypsin has a specific effect on several reactions of the electron transport chain as well as the energy-coupling system. These are the coupling factor for cyclic photophosphorylation, the water-splitting system, the link between the two photosystems, the dichlorophenylindophenol inhibition of Photosystem II and the ferredoxin NADP reductase. The ferredoxin NADP reductase appears to be one of the most exposed membrane components, since it is the only function inactivated by the soluble as well as by the insolubilized trypsin, which removes certain membrane proteins as shown by acrylamide gel electrophoretic analysis (21). From immunological experiments, Berzborn has also concluded that the ferredoxin NADP reductase is located on the outer surface of the spinach chloroplast membrane (22, 23). In agreement with these findings, the inactivation of the NADP photoreduction by trypsin appears to be due to digestion of the ferredoxin NADP reductase as shown also by the inactivation of its diaphorase activity. Selman and Bannister (5) concluded, on the basis of fast inhibition of NADP photoreduction by trypsin which was found to be reversible by addition of trypsin inhibitor, that the electron transport from the primary acceptor of Photosystem I to ferredoxin NADP reductase is inhibited by unspecific binding of trypsin on the membrane surface. The results obtained in this work, however, are not due to such a nonspecific effect, since the inactivation is time dependent, cannot be reversed by trypsin inhibitor, and was reversed by addition of the soluble enzyme.

The water-splitting activity of Photosystem II is only partially exposed to trypsin acting on the outer surface but not on the inner surface of the membrane. This conclusion is based on the findings that: (a) insolubilized trypsin does not affect the photoreduction of dichlorophenylindophenol using water as an electron donor; (b) soluble trypsin acting from within the thylakoid has no effect on the water-splitting activity under conditions in which major membrane proteins are digested (21); (c) soluble trypsin partially inactivates photoreduction of dichlorophenylindophenol with water as electron donor. The possibility that this partial inactivation might be due to trypsin digestion of the dichlorophenylindophenol-reducing site can be disregarded since Tris-treated membranes can still reduce dichlorophenylindophenol with diphenylcarbazide as an electron donor at rates similar to those obtained with water as an electron donor (Table I), and this activity is not affected by treatment with trypsin. These results agree with the
observations of Braun and Govindjee (24) and Radunz and Schmid (25), who showed that antibodies against Photosystem II particles or lutein do not completely block the water-splitting activity in spinach chloroplasts, and suggested that the membrane components responsible for this activity are protected or partially buried within the membrane rather than being exposed, as suggested by Giaquinta et al. (26).

The dichlorophenyldimethylurea-inhibition site of Photosystem II, which is known to be close to the quencher Q (27), can be affected by digestion of the membranes with soluble but not insolubilized trypsin, indicating that the reducing site of Photosystem II is exposed. Experiments being performed aimed at distinguishing between the possibilities that loss of dichlorophenyldimethylurea inhibition is due to a reduction in the binding affinity or due to a direct effect on the dichlorophenyldimethylurea-inhibitory site proper. That protein components of the reducing site of Photosystem II are reached on the membrane surface by soluble trypsin is also indicated by its effect on the link between the two photosystems and on ferricyanide photoreduction. Although, in our preparation, both dichlorophenolindophenol and ferricyanide accept electrons from Photosystem II before plastoquinone, trypsin treatment has a greater effect on the reduction of ferricyanide and interconnection of the two photosystems than on that of dichlorophenyldimethylurione when using the same electron donor. This can be explained if one assumes that these substances accept electrons at two distinct sites. Thus, dichlorophenolindophenol is more lipophilic and might react with a site located within the lipid phase, whereas ferricyanide, which is a more hydrophilic substance, might react with a protein exposed to the aqueous medium. The accessibility of the Photosystem II reducing site to trypsin acting on the outer surface of the thylakoid membrane is in agreement with other data in the literature. Strotmann et al. (7) suggested a trypsin-inhibition site between the two photosystems, and immunological tests reported by Radunz and Schmid (25) showed that plastoquinone can be reached in the membrane by its antibody.

The results presented in this work demonstrate that trypsin can be used as a tool for the tentative localization of photosynthetic activities within the membrane frame. It is proposed that in a future work, the conditions of controlled digestion as utilized in this work will be used in combination with analysis by acrylamide gel electrophoresis of membrane proteins. It is hoped that in this way, it would be possible to identify and localize trypsin-sensitive membrane proteins and to study changes in membrane organization during different stages of development expressed as changes in sensitivity toward trypsin digestion of different membrane proteins.

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