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

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Location of electron transport chain components in chloroplast membranes of *Chlamydomonas reinhardtii*, 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. Photosynthetically 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 mutants 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 reinhardtii*, y-1 has been studied.

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**MATERIALS AND METHODS**

**Isolation of Thylakoid Membranes—** *Chlamydomonas reinhardtii*, 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 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 KCl (Tris-KCl buffer) at a final concentration of 10 to 100 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-KCl buffer.

**Tris Treatment, Sonication, and Trypsin Digestion of Membranes—** Chloroplast membranes were treated with 0.5 mM Tris buffer, pH 8.5, 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 sonifer 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 dichlorophenolindophenol1 (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 dichlorophenyldimethylurea, 5 mM sodium ascorbate, and 0.1 mM dichlorophenolindophenol were measured polarographically at 25°C. Illumination was provided by incandescent light (2.5 x 10² watts/cm²). The chlorophyll concentration was between 10 and 30 μg/ml of Tris-KCl buffer.

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1The abbreviations used are: dichlorophenolindophenol, 2,6-dichlorophenolindophenol; dichlorophenyldimethylurea, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; diphenyl carbazide, 1,5-diphenyl-carbazide, Tricine, N-tris(hydroxymethyl)methylglycine.
The photoreduction of dichlorophenolindophenol (0.6 mM) by di-
phenylcarbazide (1 mM) in Tris-treated membranes (10 μg of chloro-
phyl/ml) was measured spectrophotometrically in 30 mM phosphate
buffer, pH 6.4, using an Amino-Choice dual wavelength spectropho-
tometer 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 RC 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 beams 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
photomultiplier.

The reaction mixture contained 40 mM Tricine-KOH buffer, pH 8.0;
60 mM KCl; 100 mM sucrose; 0.4 mM NADP; 3.3 μM ferredoxin; excess
amounts of ferredoxin NADP reductase when reductase, and 50 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 diuron, 0.1 mM diuron, 0.1 mM diuron,
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 photooxi-
dation 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 RKB Ultrum 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 Euge-
la gracilis was kindly given by Dr. E. Tel-Or. Soybean trypsin
inhibitor type I1, soluble trypsin from bovine pancreas (crystalized
twice), and acrylamide-bound trypsin (insolubilized) were purchased
from Sigma and Co. One unit of the insolubilized enzyme is defined as
the amount which will hydrolyze 1 μmol of N-benzoyl-L-arginine-ethyl
ester at pH 8.0 in 1 min and is equivalent to the activity of about 20 μg
of soluble trypsin. Cadmium-free ferritin was purchased from Pentex
and washed by centrifugation before use. All the reagents and solvents
used in this work were of analytical grade.

RESULTS

Digestion of membranes with trypsin was carried out at 10°,
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—Photore-
duction for ferricyanide and dichlorophenolindophenol 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 plastoqui-
none (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
dichlorophenolindophenol 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 dichlorophenolindophenol using the same
electron donor system (water) indicated that differences exist
between the ferricyanide and dichlorophenolindophenol-reduc-
sing sites, the former being more sensitive to trypsin.

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

The light-dependent reduction of dichlorophenolindophenol is
sensitive to dichlorophenolindophenol and to o-phenanthro-
line, both specific inhibitors of Photosystem II. However,
treatment of the membranes with trypsin abolished the inhibi-

Fig. 1. Inactivation of NADP photoreduction and cyclic photophos-
phorylation by trypsin as a function of enzyme concentration. The
100% rates were 61 μmol of NADPH/mg of chlorophyll/hour with
water as electron donor, and 224 μmol of ATP/mg of chlorophyll/hour
with phenazine methosulfate as cofactor.

Fig. 2. Sensitivity of Photosystem II reactions to increasing concen-
trations of trypsin. The 100% rates were 40.8 μmol of O2/mg of
chlorophyll/hour, and 38 μmol of O2/mg of chlorophyll/hour evolved
with dichlorophenolindophenol and ferricyanide, respectively.
Trypsin Sensitivity of Photosystem I Reactions and Electron Transport between the Two Photosystems—Photoreduction of NADP or methyl viologen using reduced dichlorophenolindophenol electron donor measures Photosystem I activity. Whereas photoreduction of NADP is readily inactivated by treatment with trypsin (Figs. 1 and 4), photoreduction of methyl viologen appears to be insensitive to proteolytic digestion (Fig. 4). This would indicate that the trypsin acts at the ferredoxin NADP reductase level. In order to test this possibility, the purified reductase was added to trypsin-digested membranes. Addition of reductase restores the photoreduction of NADP after trypsin inactivation (Fig. 4). Photooxidation of cytochrome f was also found to be unaffected by trypsin.

The integrity of the electron transport chain between Photosystems I and II following trypsin digestion can be tested by measuring photoreduction of NADP using electron donors specific to Photosystem II. Electron transport from diphenylcarbazide to NADP is abolished by treatment of membranes with low concentrations of soluble trypsin (Fig. 4). The inactivation of electron transport between the two photosystems is not due to digestion of the reaction centers as demonstrated in Figs. 3 and 4.

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 I). The results shown in Table I 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 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 I). 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, 10⁵⁵), followed by addition of trypsin inhibitor (100 μg/ml). c, Ferritin added during sonication (20 s, 10⁵⁵) 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 10⁵⁵, 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. (b) 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. Cytochrome f photoreduction 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 dicyanophenylindophenol reduction by trypsin acting from both sides of the membrane.

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 dicyanophenylindophenol 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 by trypsin acting from both sides of the membrane. 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. Cytochrome f photoreduction was found to be resistant to trypsin acting from both sides of the membrane.

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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 dichlorophenyl dimethylurea-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 are being performed aimed at distinguishing between the possibilities that loss of dichlorophenyl dimethylurea inhibition is due to a reduction in the binding affinity or due to a direct effect on the dichlorophenyl dimethylurea-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 dichlorophenolindophenol 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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