Biochimica et Biophysica Acta, 292 (1973) 566–581

BBA 46511

TRYPSIN INHIBITION OF ELECTRON TRANSPORT

BRUCE R. SELMAN, THOMAS T. BANNISTER and RICHARD A. DILLEY

Department of Biology, University of Rochester, Rochester, N.Y. and Department of Biological Sciences, Purdue University, West Lafayette, Ind. (U.S.A.)

(Received October 6th, 1972)

SUMMARY

1. A relaxation spectrophotometer was employed to measure the effects of trypsin treatment on electron transport in both cyclic and non-cyclic chloroplast reactions. The parameters measured were electron flow rate through P700 (flux) and the time constant for dark reduction of P700.

2. In the reduction of methyl viologen by the ascorbate–2,6-dichlorophenolindophenol (DCIP) donor couple, there was no effect of trypsin on P700 flux or on the time constant for dark reduction of P700. In the phenazine methosulfate (PMS) cyclic system, trypsin had either a slightly stimulatory or slightly inhibitory effect on the P700 flux, depending on the presence or absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU): either effect being marginal compared to trypsin effects on Photosystem II.

With both ferricyanide and methyl viologen reduction from water, trypsin treament gave a first order decline in P700 flux: which matched the trypsin-induced decline in electron transport with the water to DCIP system, measured by dye reduction. This implies that Photosystem II is inhibited. The inhibition of Photosystem II was up to 90% with a 6–10-min trypsin treatment. This result is consistent with the concept of Photosystem I (P700) being in series with Photosystem II in the electron transfer sequence.

3. Cyclic phosphorylation was severely inhibited (85%) by trypsin treatment which had a somewhat stimulatory effect on P700 flux, indicating uncoupling. Non-cyclic phosphorylation was uncoupled as well as electron flow being inhibited since the P/2*e* ratio decreased more rapidly as a function of trypsin incubation time than inhibition of electron flow. The two effects, uncoupling and non-cyclic electron flow inhibition, are separate actions of trypsin. It is probably that the uncoupling action of trypsin is due to attack on the coupling factor protein, known to be exposed on the outer surface of thylakoids.

4. Trypsin treatment caused an increase in the rate constant, k_d, for the dark H^+ efflux, resulting in a decreased steady state level of proton accumulation. The increased proton efflux and the inhibition of phosphorylation are consistent with an uncoupling effect on trypsin.

5. Trypsin treatment did not reduce the manganese content of chloroplasts:

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; Tricine, *N*-tris(hydroxymethyl)methylglycine; MES, 2-(*N*-morpholino)ethanesulfonic acid; DCMU, (3,4-dichlorophenyl)-1,1-dimethylurea.

as reported by others, Tris washing did remove about 30% of the chloroplast manganese.

6. Electron micrographs of both negatively stained and thin-sectioned preparations showed that, under these conditions, trypsin does not cause a general breakdown of chloroplast lamellae. Inhibition by trypsin must therefore result from attacks on a few specific sites.

7. Both System II inhibition and uncoupling occur rapidly when trypsin treatment is carried out in dilute buffer, a condition which leads to thylakoid unstacking, but both are prevented by the presence of 0.3 M sucrose and 0.1 M KCl, a condition that helps maintain stacked thylakoids. Evidently vulnerability to trypsin requires separation of thylakoids.

8. Since trypsin does not appear to disrupt thylakoids nor prevent their normal aggregation in high sucrose–salt medium and since the trypsin molecule is probably impermeable, it is probable that the site(s) of trypsin attack in System II are exposed on the outer thylakoid surface.

INTRODUCTION

Energy-transducing membranes can be experimentally manipulated using a protease such as trypsin. By hydrolysing the peptide chain only at lysine or arginine residues, and by being rapidly inhibited upon addition of trypsin inhibitor, this enzyme treatment can have fairly mild but specific effects. It is especially useful as a tool in studying the structural arrangement of various parts of the membrane, since it is a large, water-soluble enzyme restricted to acting on membrane proteins exposed to the bathing medium. To be so used in the chloroplast system the effect of trypsin on various biochemical reactions of chloroplast membranes must be clearly documented. It is clear from the work of Selman and Bannister[3] that trypsin can inactivate water oxidation more rapidly than the photochemical steps of Photosystem II, since alternate electron donors can be effectively oxidised with the treated membranes. On the other hand, Mantai's[2] and our results[3] indicate that electron flow through Photosystem I is not directly affected by trypsin, using the ascorbate–2,6-dichlorophenolindophenol (DCIP)→NADP[2] or the ascorbate–DCIP→methyl viologen[3] system as criteria. These same authors also noted a trypsin-induced transient acceleration of electron flow from water to Photosystem I acceptors, with the effect being abolished by uncouplers. Both groups concluded that uncoupling of electron flow from phosphorylation was occurring. A different view was recently put forward by Strotman *et al.*[1]. In their studies of cyclic phosphorylation with phenazine methosulfate (PMS), trypsin inhibited phosphorylation and, in addition, slowed the development of the pH gradient when light was turned on, but did not affect the decay kinetics when light was turned off. Strotman *et al.*[1] reasoned that an uncoupler should accelerate the decay (which was not observed) while electron transfer inhibitors would slow the initial rise (which was observed), and they therefore concluded that trypsin acts not as un uncoupler but rather as a cyclic electron transfer inhibitor.

As a critical evaluation of the differences in reported trypsin effects, we have measured the electron flow rate through Photosystem I (P700 turnover) and the rate constant for the dark reduction of P700 by using the relaxation spectrophotometer

where ω is the frequency of modulated actinic beam and ϕ is the phase angle. The flux through P700 can be calculated from the equation:

$$v_{700}\ (\mu\text{equiv/mg chlorophyll per h}) = \frac{A}{\varepsilon\tau \cos\theta}$$

where A is the amplitude of the modulated signal and ε is the extinction coefficient of P700 (assumed to be 65 $cm^{-1} \cdot mM^{-1}$ (ref. 12)). Rurainski[11] has tested these assumptions and has shown that P700 flux can be reliably calculated.

The Mn^{2+} content of chloroplasts was determined as described by Heath and Hind[13]. After either washing with 0.8 M Tris–HCl (according to the method of Yamashita and Butler[14]) or after trypsin treatment (for 10 min) suspensions were centrifuged at $12000 \times g$ for 20 min and the chloroplasts resuspended in 1–2 ml of buffer containing 10 mM Tris–HCl (pH 7.6) and 10 mM NaCl. To 1.5 ml of chloroplasts, containing 1–3 mg chlorophyll, 0.5 ml of a mixture of 17 parts (70%) HNO_3 to 3 parts (70%) $HClO_4$ (v/v) was added. The suspension was centrifuged at low speed for 20 min, the supernatant decanted, the pellet resuspended in 2.0 ml of water, and the suspension centrifuged. The combined supernatant fractions were centrifuged again for 20 min and then aspirated directly into a Perkin–Elmer Model 303 atomic absorption spectrophotometer. Sensitivity (greater than 2 μM Mn^{2+} final concentration) was sufficient to make extraction into methyl isobutyl ketone unnecessary.

Light-induced pH changes and electron transport were measured as before[15] and provided information about the activity of the proton transport, the rate of ferricyanide reduction, and the rate of PMS-catalyzed cyclic photophosphorylation. For these studies, trypsin-treated chloroplasts were centrifuged as $12000 \times g$ for 10 min and resuspended in buffer containing either 1 mM *N*-tris(hydroxymethyl)methylglycine (Tricine) or 0.11 mM 2-(*N*-morpholino)ethanesulfonic acid (MES). Proton influx and efflux apparent first order rate constants were determined by the usual graphical techniques. Using the Karlish and Avron[27] approach, we computed the proton binding capacity YH_0 from the rate constants and the observed steady-state proton accumulation, $YH^+{}_{2ss}$.

Electron microscopy was carried out as previously described[16]. Negative staining was achieved by mixing equal volumes of 2% phosphotungstic acid and chloroplast suspension on a carbon over Formvar-coated grid, then removing the excess liquid with filter paper. For thin sectioning, samples were fixed in 2% glutaraldehyde, postfixed in 2% OsO_4, embedded in Epon, and post-stained with uranyl acetate and lead citrate. Micrographs were taken on a Phillips Model 300 electron microscope.

RESULTS

P700 flux and relaxaton time

In order to show that trypsin inhibition of electron transport can be studied by measuring either net reduction or flux through P700, we compared the decline of net reducing activity in the DCIP reaction measured by dye reduction with the decrease in P700 flux in the methyl viologen-catalysed Mehler reaction. P700 flux associated with net electron transport cannot be measured in the reaction water to DCIP because DCIP also catalyses cyclic electron transport in Photosystem I.

This does not happen in either the methyl viologen or ferricyanide Hill reaction. Fig. 1. shows typical results. Both electron transport and P700 flux were found to decline with identical kinetics. Since the site of inhibition of electron transport is on the oxidizing side of Photosystem II (ref. 3) while System I is unaffected by trypsin (ref. 3 and this paper), it is evident that the flux can be used to measure the inhibition of electron transport by trypsin.

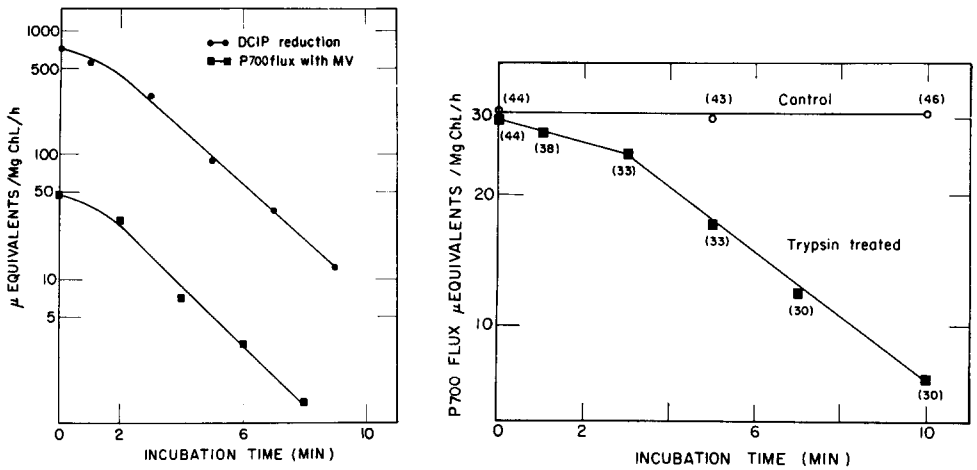

Fig. 1. Effect of trypsin on electron transport and P700 turnover. Chloroplasts were prepared and trypsin treated as described in Methods. Reaction mixtures for DCIP reduction contained in 3.0 ml chloroplasts, 10 μg chlorophyl/ml; DCIP, 20 μM; Tris–HCl, 10 mM (pH 7.6); NaCl, 10 mM and methylamine·HCl, 33 mM. Incident illumination about $5 \cdot 10^5$ ergs/cm^2 per s (see ref. 3). Reaction mixtures for P700 turnover contained in 2.0 ml chloroplasts, 10 μg chlorophyll/ml; methyl viologen (MV), 0.5 mM; Tris–HCl, 10 mM (pH 7.6); NaCl, 10 mM and methylamine, 33 mM. ChL, chlorophyll.

Fig. 2. Semi-log plot of P700 flux in the presence of ferricyanide as a function of duration of trypsin treatment. Numbers in parentheses are relaxation times (τ) in ms. Reaction mixtures of 2.0 ml volume contained chloroplasts, 10 μg chlorophyll/ml; ferricyanide, 1 mM; Tris–HCl, 10 mM (pH 7.6) and NaCl, 10 mM.

Fig. 2 shows the effect of trypsin on P700 flux associated with the ferricyanide Hill reaction with water as donor. Trypsin treatment inhibited the flux through P700; after a brief induction period, the flux decreased with first order kinetics with respect to incubation time with trypsin. The first-order decrease in rate was always seen but the half-time ranged from 1.5 to 3.0 min among different chloroplast preparations. Trypsin treatment also caused the P700 relaxation time, *i.e.* the time constant for dark reduction of P700 (numbers in parentheses), to decrease rapidly during the initial induction period: thereafter, and continuing throughout most of the period of declining flux, the relaxation time remained constant.

Table I shows that a trypsin treatment which inhibited 90% of the P700 flux associated with ferricyanide reduction (using water as donor) had a negligible effect on P700 turnover in the reaction mediated by PMS. Fig. 3 shows similar results for P700 flux associated with methyl viologen reduction. With water as donor, P700 flux again declined with first-order kinetics with respect to incubation time with

TABLE I

Spinach chloroplasts were prepared and trypsin treated as described in Methods. Reaction mixtures contained in 2.0 ml, chloroplasts, 13.5 μg chlorophyll/ml; Tris-HCl, 10 mM (pH 7.6); NaCl, 10 mM; DCMU (where indicated), 10 μM and either PMS (concentration as indicated) or ferricyanide, 1 mM.

	P_{700} flux (μequiv/mg chlorophyll per h)				
	Concentration of PMS (μM):				*Ferricyanide*
	7.5	*15*	*30*	*150*	
	— +DCMU	*— +DCMU*	*— +DCMU*	*— +DCMU*	
Control	22 40	20 37	15 31	17 14	31
Treated (5 min)	16 38	17 35	22 25	11 18	8
Treated (10 min)	24 33	31 31	24 20	15 16	3

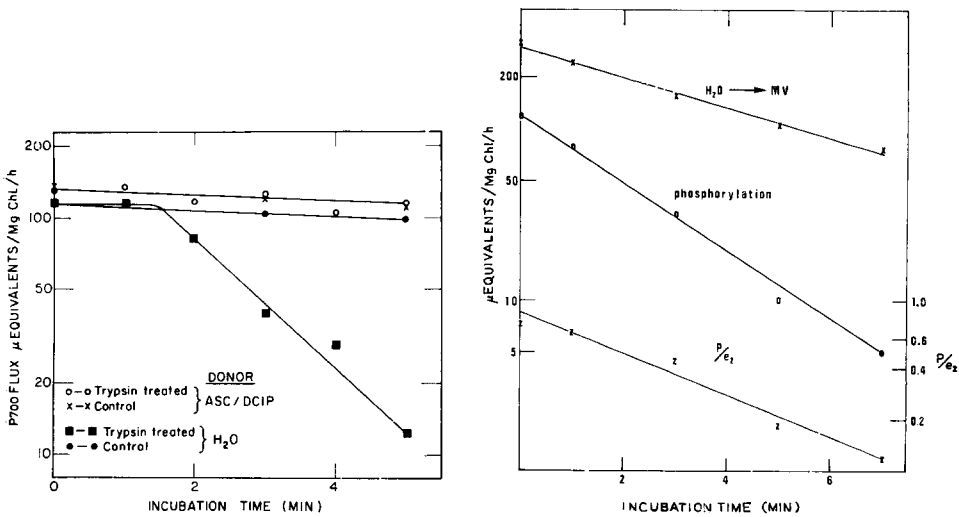

Fig. 3. Effect of trypsin on P700 flux with methyl viologen as electron acceptor. Conditions as described in Methods. Tryptophan/chlorophyll ratio during incubation was 0.13. Reaction mixture contained in 2.0 ml chloroplasts, 10 μg chlorophyll/ml; methyl viologen, 0.5 mM; Tris-HCl, 10 mM (pH 7.6) and NaCl, 10 mM. The upper curve(s) contained in addition DCMU, 10 μM; DCIP, 0.1 mM and ascorbate (ASC), 1 mM.

Fig. 4. Effect of trypsin on non-cyclic phosphorylation and electron transport. Electron transport to methyl viologen was determined from the steady state rate of oxygen consumption using a Clark-type electrode (see ref. 3). At the same time, phosphorylation was determined from the steady state rate of pH increase (due to formation of ATP (see ref. 15) in the weakly buffered reaction mixture. Chloroplasts were prepared and treated as described in Methods. Following trypsin treatment, 15-ml suspensions were centrifuged at 12000 $\times g$ for 10 min and the pellet resuspended in 1 ml of weak buffer contaning Tricine, 1 mM; $KHPO_4$, 3 mM and KCl, 0.1 M. Reaction mixtures contained in addition methyl viologen (MV), 0.5 mM; KCN, 1 mM; $MgCl_2$, 5 mM; Chloroplasts, 30–40 μg Chlorophyll/ml and ADP, 1 mM in a total volume of 5.0 ml. After adjusting the pH to 8.1 $\pm$ 0.05 with 0.1 M KOH, the mixture was illuminated with white light carefully freed of infrared. Suspensions were maintained at 15 °C. Changes in pH and concentration of dissolved oxygen were recorded on a Mosely two pen strip chart recorder. Changes in pH were calibrated at the end of the reaction by the addition of 10 μl 0.01 M HCl.

trypsin, but there was no inhibition with the ascorbate–DCIP donor system in the presence of 3-(3,4-dichlorophenyl)-1,1 dimethylurea (DCMU). These observations confirm that trypsin inhibits electron transport in System II but not System I. Apparently none of the proteins essential to electron transport in System I are exposed or susceptible to trypsin hydrolysis.

Phosphorylation

The increase in the rate constant (*i.e.* decrease in time constant) for the re-reduction of P700 (Fig. 2) in the non-cyclic reaction is consistent with trypsin acting as an uncoupler at or close to System I. In a similar experiment to that shown in Fig. 2, the uncoupler methylamine was included in the reaction mixture, and the time constant was reduced to a range of 21 to 26 ms for controls and trypsin treatments alike. The time constant for the controls without uncoupler were similar to those shown in Fig. 2, *i.e.* around 45 ms. Again a similar first order decline of P700 flux was observed over the 10-min trypsin treatment, with the time constant remaining from 21–25 ms. These results, along with previous ones[2] showing that a short trypsin treatment stimulated the light-saturated Hill reaction with DCIP, provide strong evidence that trypsin uncouples non-cyclic electron transport from phosphorylation. Additional evidence is provided in Fig. 4. which shows the results of simultaneous measurements of phosphorylation (by the steady-state pH change) and of electron transport (as oxygen consumption) in the methyl viologen Hill reaction. Both processes are inhibited with first order kinetics, but the inhibition of phosphorylation is the more rapid. As a consequence, the P/e_2 ratio also drops with apparent first-order kinetics.

TABLE II

Spinach chloroplasts were prepared and trypsin treated as described in Methods. After treatment the chloroplasts were centrifuged and resuspended in Tricine, 1 mM; $MgCl_2$, 5 mM; NaCl, 100 mM and K_2HPO_4, 3 mM. Assay conditions for measuring proton fluxes were: KCl, 100 mM; $MgCl_2$, 5 mM; PMS 0.03 mM and 33 μg chlorphyll/ml with the initial pH adjusted to 7.5–7.6. The numbers in parentheses in the table refer to the percent of the control. Phosphorylation with PMS was measured in separate experiments using a similar reaction mixture to that above with the addition of 3 mM K_2HPO_4 and 1 mM ADP and with the pH adjusted to 8.0–8.1. White light was passed through a Corning I-69 infrared filter. The temperature was 15 °C.

Cofactor	*Trypsin treatment*	*Rate constants (s^{-1}) for H^+ fluxes*			*Extent of H^+ accumulation (μmoles H^+/ mg chlorophyll)*	*ATP formation (μmoles ATP/ mg chlorophyll per h)*
		$k^*_{(obs)}$	k_d	k_f		
PMS	Control	0.12	0.094	0.21	0.3 (100)	462 (100)
PMS	3 min	0.13	0.144	0.28	0.16 (52)	—
PMS	5 min				0.14 (46)	197 (42)
PMS	10 min				—	75 (16)

*k_f is the true forward constant, computed as the sum of the observed forward rate constant and the decay constant, *i.e.* $k_f = k_{f(obs)} + k_d$.

Table II shows that trypsin treatment also inhibits cyclic phosphorylation with PMS, in agreement with the results of Strotman *et al.*[1]. Since P700 flux is not inhibited in the absence of DCMU (under the conditions for the ATP formation assays, P700 flux is stimulated, Table I), we conclude that trypsin uncouples cyclic as well as non-cyclic phosphorylation. The trypsin-induced decrease in cyclic phosphorylation was also first order with incubation time.

Proton fluxes

Table II summarizes data obtained for the effects of trypsin on the forward (k_f) and decay (k_d) apparent first-order rate constants, and the extent of H^+ accumulation observed when weakly buffered chloroplast suspensions were illuminated. The rate constants for the PMS-catalysed reaction were calculated from the slopes of semi-log plots of the forward or decay phases of pH change. The true forward rate constant is assumed to be the sum of the observed rate constant, $k_{f(obs)}$, and the decay constant, k_d[25,27]. The decay rate constant is increased about 150% by the trypsin treatment, with little effect on the observed forward rate constant. This explains the lowering of the steady-state H^+ accumulation found in the trypsin-treated sample. The lack of inhibition of the inward H^+ flux by trypsin treatment agrees with the measurements of P700 flux, which showed only a slight change in P700 turnover in the PMS case (Table I). Strotman *et al.*[1] reported that trypsin treatment caused a decrease in the steady-state proton accumulation with no change in the $t_{\frac{1}{2}}$ of the decay phase. Under our conditions this clearly was not the case.

Trypsin treatment did not alter the total proton-binding capacity of the membranes. This is indicated by kinetic analysis following Karlish and Avron[27]. Their treatment defines the total proton binding capacity, YH_0, and the steady-state observed proton binding, YH_{2^+ss}, and relates these parameters to the k_d and $k_{f(obs)}$ by the equation below.

$$YH_0 = \frac{k_{f(obs)} + k_d}{k_{f(obs)}} YH_{2^+ss}$$

Using the measured parameters we calculate that YH_0 is 0.59 μmole H^+/mg chlorophyll for the control PMS case, and 0.60 μmole H^+/chlorophyll mg for the trypsin treatment.

Mn^{2+} content of spinach chloroplasts

Table III summarizes the Mn^{2+} content of chloroplasts after washing with 0.8 M Tris–HCl (pH 8.0) or after trypsin treatment. It was shown that both treatments inhibited by more than 90% the DCIP Hill reaction of untreated controls. Our values of Mn^{2+} content in untreated chloroplasts (50–100 moles chlorophyll per gatom Mn^{2+}) agree with results previously reported for spinach chloroplasts[5,17]. That Tris washing removed 30–40% of chloroplast Mn^{2+} is in good agreement with Itoh *et al.*[5]. Table III clearly shows that trypsin treatment does not reduce the manganese content of chloroplasts and it may, therefore, be concluded that Mn^{2+} removal is not the mechanism of inhibition of oxygen evolution by trypsin.

Effect of incubation mixture on trypsin inhibition of electron transport

Routinely, we have carried out trypsin incubation in a medium containing 10 mM Tris and 10 mM NaCl[3]. In this medium 50% inhibition occurs in about 2–4

TABLE III

Mn^{2+} CONTENT OF SPINACH CHLOROPLASTS

Chloroplasts were prepared and treated as described in Methods. Tryptophan/chlorophyll ratio during incubation was 0.13. After treatment with either trypsin or Tris, the rate of the DCIP Hill reaction was less than 10% of the control.

Expt	*Chlorophyll/Mn^{2+} ratio*		
	Control	*Trypsin treated*	*Tris washed*
1	93	—	120
2	74	—	101
3	51	—	85
4	92	98	—
5	47	46	—
6	51	55	—
% Mn^{2+} (av.)	100	96	70

min. When both 0.3 M sucrose and 0.1 M KCl were also included, inhibition by trypsin was greatly slowed. Either 0.3 M sucrose or 0.1 M KCl alone protected chloroplasts from trypsin to some extent, but better protection required both compounds together.

Fig. 5 shows the results of an experiment in which conditions were changed

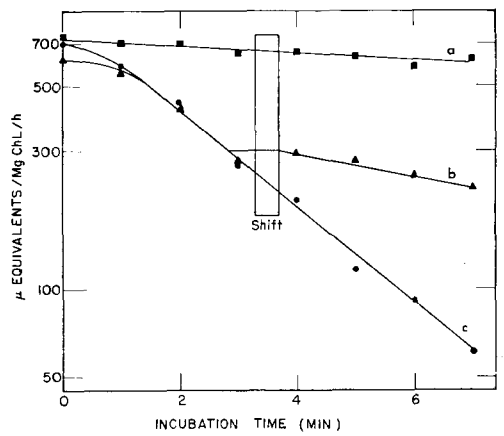

Fig. 5. The effects of shifts in incubation conditions on trypsin inhibition of DCIP reduction. Spinach chloroplasts were prepared and treated as described in Methods. Tryptophan/chlorophyll ratio during incubation was 0.13. The initial incubation mixture contained chloroplasts, 60 μg chlorophyll/ml in buffer composed of Tris–HCl, 10 mM (pH 7.6) and NaCl, 10 mM. Curve a contained in addition sucrose, 0.3 M and KCl, 0.1 M. At approx. 3.5 min (as indicated) the incubation mixtures were diluted (shifted) with buffer to contain in final concentration chloroplasts, 30 μg chlorophyll/ml in buffer composed of Tris–HCl, 10 mM (pH 7.6) and NaCl, 10 mM and (Curves a and b only) sucrose, 0.3 M and KCl, 0.1 M. Reaction mixtures for DCIP reduction contained in 3.0 ml chloroplasts, 10–20 μg chlorophyll/ml; Tris–HCl, 10 mM (pH 7.6); NaCl, 10 mM; DCIP, 20 μM and methylamine, 33 mM. Reactions were run at 18 °C and incident illumination was about $4 \cdot 10^5$ ergs/cm^2 per s.

during trypsin incubation. In Curve 5a, chloroplasts suspended in sucrose–KCl were diluted by adding additional sucrose–KCl solution. The inhibition proceeded very slowly ($t_{\frac{1}{2}} > 20$ min) and was not affected by the dilution. Curve 5c shows chloroplasts suspended in dilute buffer (10 mM Tris–HCl, pH 7.6, and 10 mM NaCl) and diluted with the same buffer. Trypsin inhibition occurred rapidly ($t_{\frac{1}{2}} = 1.7$ min) and was also unaffected by the dilution. Curve 5b shows that the inhibition is abruptly slowed when chloroplasts, initially in dilute buffer, were diluted with sucrose–KCl. Before the shift, the half-time for inhibition was 1.7 min while, after the shift, the half-time increased to about 10 min. The same result could be obtained at any time during the incubation, from 1 min out to 4.5 min, as shown in other experiments.

Electron microscopy

Izawa and Good[8] and Anderson and Vernon[18] have shown that, in low salt buffers, the grana stacks of chloroplasts disappear and that the lamellar membranes appear as individual thylakoids. Trypsin incubation was routinely carried out in low salt (*i.e.* in hypotonic Tris buffer containing 10 mM NaCl, see ref. 3). Fig. 6A shows a typical electron micrograph of thin-sectioned control chloroplasts. As expected the isolation and incubation procedures disrupt the chloroplasts and result in loosely associated lamellae characteristic of low salt. Fig. 6B shows that lamellar membranes from chloroplasts treated with trypsin for 10 min have the same appearance as untreated controls.

Electron micrographs of negative-stained chloroplasts indicated no significant differences between control and trypsin-treated chloroplasts. In both cases, 90–110-Å particles (probably chloroplast coupling factor 1 and carboxydismutase[16]) were present on the membranes with the same density distribution. The appearance of the membranes was similar in both control and trypsin treatments again indicating that under these conditions, no general disruption of the membrane occurred.

DISCUSSION

With the non-cyclic electron transfer system, our results for P700 flux and relaxation times (Figs 1–3) are consistent with the reported effects of trypsin on the rate of DCIP reduction[2,3]. With water as donor, the decrease of P700 flux, both in the presence and absence of an uncoupler, is most easily explained as due to attack by trypsin on a site between water oxidation and 1,5-diphenylcarbohydrazide oxidation[3]. The rapid decrease of τ (*i.e.* an increase in the rate constant for the dark reduction of $P700^+$) is only observed in the absence of an uncoupler and indicates that trypsin decreases the relaxation time by uncoupling. Furthermore, although trypsin decreases τ from 45 tot 30 ms in coupled chloroplasts (Fig. 2), the relaxation time is not as short as when an uncoupler is included in the reaction mixture (a τ of about 25 ms, see Results). This is perhaps confirmation of Mantai's[2] report that trypsin only partially uncouples spinach chloroplasts.

That the observed inhibition of non-cyclic electron flow from water is not due to trypsin inhibition of Photosystem I electron transfer is shown in Fig. 3 and Table I. When P700 flux in the ferricyanide Hill reaction is inhibited about 90% (Table I) by a 10-min trypsin treatment, P700 flux in the PMS cyclic system is either stimulated up to 150%(in the absence of DCMU) or inhibited by 30% (in the presence of DCMU).

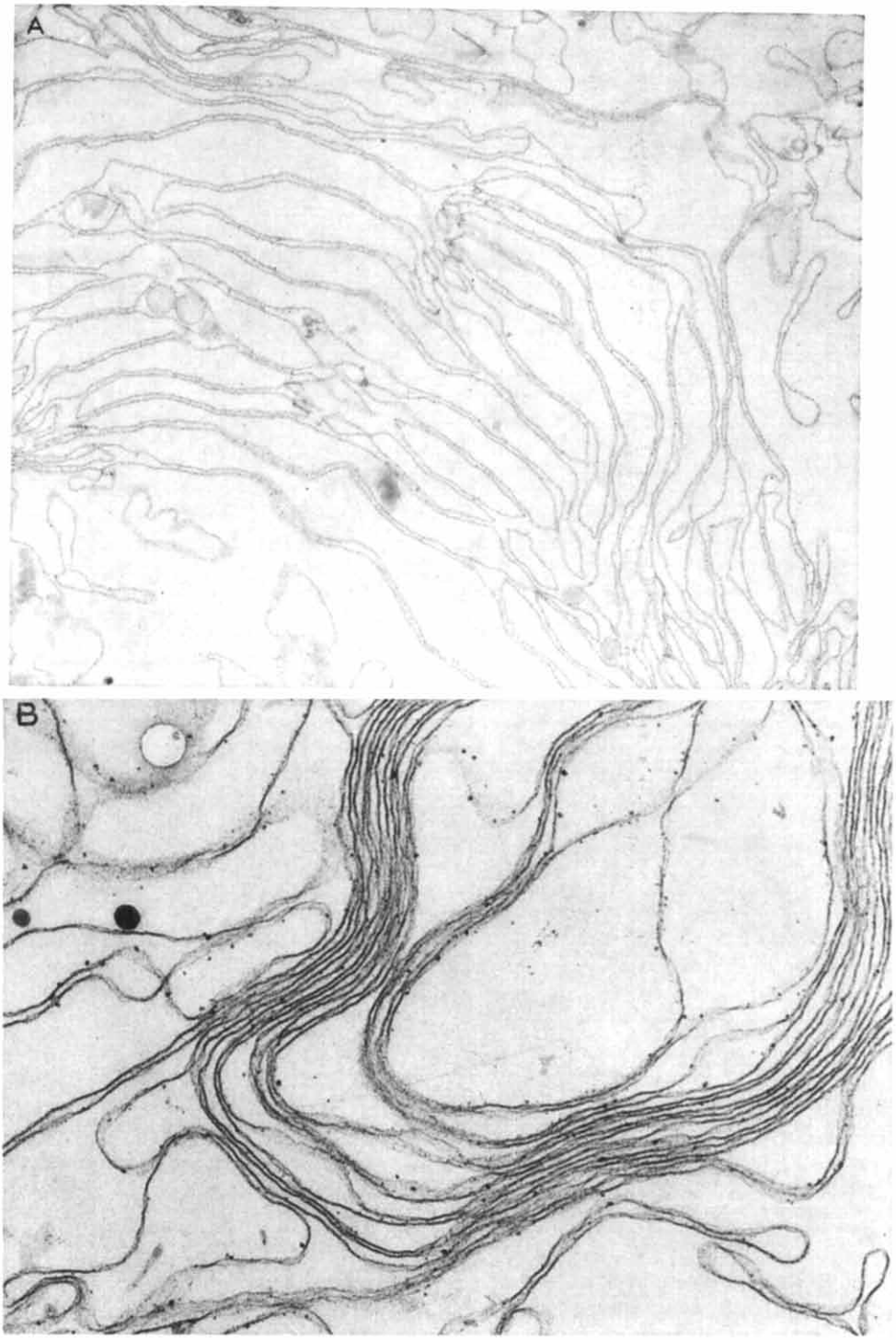

Fig. 6. Electron micrographs of thin-sectioned chloroplasts. Spinach chloroplasts were treated and prepared for electron microscopy as detailed in Methods. (A) Control chloroplasts ($\times$ 29 750) and (B) trypsin-treated chloroplasts (10 min) ($\times$ 33 000).

The stimulation may be partly due to uncoupling, and partly due to trypsin inhibition of Photosystem II, mimicking an DCMU inhibition of Photosystem II. If DCMU leads to a greater PMS-mediated P700 flux, why should not other conditions which likewise inhibit Photosystem II? The inhibition of P700 turnover observed in the presence of DCMU with the 10-min trypsin treatment only occurred when the control rates were considerably higher than in the −DCMU case. This inhibition may be related to that reported by Strotman *et al.*[1], but it is a marginal effect compared to trypsin effects on Photosystem II and phosphorylation.

The close correspondence between Photosystem II inhibition and that of P700 flux is evidence for P700 being in series with Photosystem II in the electron transfer sequence. The alterate view, that P700 is not so involved is favored by Arnon *et al.*[31] who postulate that two short wavelength reaction centers are arranged in series with P700 being involved as a third photosystem associated only with cyclic electron flow. Rurainski *et al.*[33] have found discrepancies between P700 flux and $NADP^+$ reduction, such that under some conditions electron flow from water to NADP did not appear to go through P700. Ferricyanide is known to inhibit cyclic phosphorylation and therefore cyclic electron flow. However, in the ferricyanide system used here (Fig. 2 and Table I), P700 flux was, in the first place, functiong at about the same rate as with PMS cyclic electron flow (Table I), and in the second place, P700 flux was inhibited by trypsin with a similar time course as the inhibition of Photosystem II electron transfer measured by DCIP dye reduction (Fig. 1). Assuming that the Photosystem I (P700) centers measured in the methyl viologen case were also measured in the PMS case, the data are most consistent with Photosystem I being in series with Photosystem II. That some Photosystem I centers, *i.e.* those occurring in the stroma lamellae (one third of the total) which have no Photosystem II centers[32], are not in series with Photosystem II, and function as cyclic systems only, is very likely. Such stroma lamellae centers would probably not be observed to turnover in the presence of methyl viologen or ferricyanide (as they would be without a cyclic cofactor) and hence the instrument would be monitoring only those Photosystem I centers in direct communication with the Photosystem II centers.

From P700 studies, trypsin treatment does not significantly inhibit cyclic electron transport with PMS (Table I), but it does inhibit cyclic phosphorylation (Table II), and uncouples non-cyclic phosphorylation from electron transport (Fig. 4). Thus, the data from our separate experiments would predict that the P/e_2 ratio for cyclic electron transport should be decreasing with increasing trypsin treatment, a characteristic of uncoupling. Our results do not support the contention of Strotman *et al.*[1] that the inhibition of cyclic phosphorylation is due to inhibition of cyclic electron transport. Rather, the effect of trypsin on both cyclic phosphorylation and non-cyclic phosphorylation can be simply attributed to uncoupling.

The uncoupling effect of trypsin is also evident in its effect on H^+ fluxes (Table II). Kinetic analysis showed an increase of 150% in the apparent first order rate constant, k_d, for the H^+ efflux, an effect commonly associated with uncoupling[26]. The apparent forward rate constant, $k_{f(obs)}$, increased only slightly after a 3-min trypsin treatment while the true forward rate constant, $k_f = k_{f(obs)} + k_d$, increased by 133%. This increase can be accounted for by an increased flow of electrons through the cyclic pathway, an effect noted in Table I for the −DCMU case. While there was considerable scatter in the P700 flux data for the PMS case (Table I), there is

ample evidence for the increased electron flow rates required to explain the proton flux data. The increased proton efflux can account for the decrease in the steady state proton gradient observed in the PMS case. The decreased steady state proton accumulation was not due to a change in the binding capacity (YH_0 in the Karlish and Avron[27] terminology) as evidenced by the same calculated value for YH_0 (see Results) in both the control and the 3-min trypsin treatment.

These results suggest that trypsin may attack some protein involved in the generation or discharge of the so-called "high energy state". Trypsin may attack chloroplast coupling factor 1 *in situ**, and if so, that could have the observed effects both on photophosphorylation and on proton efflux, since it is known that chloroplast coupling factor 1 is somehow closely involved with the control of proton permeability [19,20]. The removal of chloroplast coupling factor 1 is attended by a large increase in k_d, as though a leak were introduced into the membrane[19]. Reconstitution of chloroplast coupling factor 1 to the membranes restores the H^+ permeability barrier and phosphorylation. Although the electron micrographs of negatively stained chloroplasts indicated that chloroplast coupling factor 1 was not removed from the membranes by the trypsin treatment[21], the trypsin effect of activating the Ca^{2+}-ATPase, may be accompanied by an increase in the H^+ efflux rate constant. In our hands, trypsin did not decrease the H^+ influx rate, but rather the true k_f was increased by 133%. Now it is possible to observe a lower $k_f(_{obs})$ due to some treatment compared to a control $k_f(_{obs})$, if the k_d is sufficiently increased, since $k_f(_{obs}) = k_f - k_d$. The discrepancy between the results of Strotman *et al.*[1] and ours on this point is considerable. At a lower trypsin concentration (0.004 mg/ml compared to 0.12 mg/ml), we find k_d for H^+ efflux significantly increased, while they report no effect on the efflux rate constant. It is hard to imagine that we would find no effect on k_d had we used their higher trypsin concentration.

Strotman *et al.* used a questionable assumption to justify their conclusion about the rate of cyclic electron flow from H^+ flux rates. They assumed a fixed stoichiometry for the H^+/e^- ratio in the cyclic system, whereas it is obvious from the literature[15,27,28] that there is no fixed stoichiometry, and that the measured ratios are, in fact, very sensitive to experimental conditions. Therefore, it does not seem reasonable to use H^+ flux data to draw critical conclusions concerning the effect of a treatment on cyclic electron flow.

In the non-cyclic reaction with methyl viologen, proton transport, like phosphorylation, is more rapidly inhibited by trypsin than is electron transfer. This is consistent with more rapid attack on chlorophyl coupling factor 1, or other proteins associated with phosphorylation and proton transport, and a somewhat slower attack on Photosystem II, which leads to inhibition of electron transport.

The mechanism of the inhibition of oxygen evolution by trypsin remains unresolved, but it is clear now that the removal of Mn^{2+} is not the cause (Table III). Trypsin inactivation is therefore not like that of Tris washing, hydroxylamine extraction, heating or aging; all of which appear to release Mn^{2+} simultaneously with inactivation[5,6]. In the case of the pronases, which do inhibit electron transport[7] and release Mn^{2+} (ref. 22), it would be of interest to determine if proteolysis might directly cause inhibition and only secondarily a loss of Mn^{2+}.

* In other experiments we have observed that trypsin treatment of chloroplast lamellae will elicit the Ca^{2+}-dependent ATPase activity.

Greenblatt *et al.*[9] showed that trypsin treatment grossly disrupted *Euglena* chloroplast structure. (Their conditions —high trypsin concentration and incubation at 37 °C— were much more severe than ours.) From this observation, Mantai[2] suggested that trypsin inhibition of electron transport might result from a general disruption of lamellae. Our electron micrographs do not support this, but rather show that trypsin-treated lamellae remain intact and are indistinguishable from untreated controls. In both cases, the low salt, low sucrose medium grossly disrupts the orderly arrangement of the lamellae. A "general disruption" of the membranes *per se* is not observed, as would be indicated by formation of small vesicles of fragmentation of thylakoids, or perhaps a marked change in the appearance of cross sections of stained membranes. This type of general membrane disruption is associated with such treatments as hard sonication, and digitonon or triton fractionation[29,30]. In those cases, H^+ accumulation is one of the most easily destroyed parameters. Since H^+ transport is readily observed, though noticeably affected, after trypsin treatment, it is apparent that this protease treatment is not leading to general disruption of the membrane structure. Indeed, it would be surprising if trypsin hydrolysis, being limited to attacks on lysine and arginine residues, and those supposedly only at the external membrane surface, would have much affect on the largely hydrophobic forces which maintain membrane structure. Furthermore, trypsin does not remove the 100-Å particles, which Howell and Moudrianakis[21] and Murakami[23] have shown include the coupling factor, chloroplast coupling factor 1. Rather than a general destruction of lamellar structure, our findings point to an attack by trypsin on a few, specific sites, at least some of which are important for function and none of which are essential for general morphological integrity of the lamellar memebranes.

Izawa and Good[8] have demonstrated that thylakoid stacking of isolated chloroplasts is determined by the concentration of salts in the suspension medium (*e.g.* in low salt, thylakoid stacks disappear while either in high salt or shifting from low to high salt the thylakoids remain tightly stacked). The protective effects of high salt and sucrose concentrations against rapid trypsin inhibition of the DCIP Hill reaction indicates that the vulnerability to trypsin is only associated with the "loose open state", not with the "tightly stacked state" of chloroplast lamellae. Apparently trypsin cannot penetrate tightly stacked lamellae. Since trypsin inhibition slows markedly when salt and sucrose are added, it would appear that, when the stacking takes place in the presence of trypsin, trypsin is either expelled or immobilized, or that the sites of attack are protectively sealed in an adjacent lamella. This kind of behavior is more readily understandable in terms of attack upon specific sites than in terms of a non-specific disruption of lamellae.

Since trypsin attacks two sites between water oxidation and System II[3] (as well as a third site associated with coupling to phosphorylation), it is reasonable to conclude that a portion of System II is exposed on the outer surface of the thylakoid membrane. Recent studies by Dilley *et al.*[24] have dealt directly with the question of the relative location of the two photosystems (as they are defined following digitonin fractionation) within the grana membrane. Their experiments, in which outer membrane surfaces were labelled with a non-penetrating diazonium compound, suggested that System I is exposed on the outer lamellar surface and that most of System II is located at the inner surface. However, a small amount of diazonium label was

always found in System II fractions. This result, as discussed before[24], could be due to a small portion of System II being exposed on the outer surface of the lamellae, available both to diazonium labelling and trypsin attack. To the extent that lamellae are correctly viewed as unperforated and semi-permeable discs, the outer surface of which can be identified by the action of an impermeable molecule, an external location of at least two sites of System II is strongly supported by the attack of the large protein molecule trypsin. These results logically suggest the hypothesis that the site for water oxidation is located close to, or at, the external membrane surface. Tests for this hypothesis are currently underway.

ACKNOWLEDGEMENTS

The authors are grateful to Mr Salil Bose who assisted in measurements of P700, Mr Steve Dodge for preparing samples for electron microscopy, and Dr G. Hoch for many helpful discussions. This work was supported by a Training Grant (GM-00658) from the National Institute of General Medical Sciences and by grants (GB-6973) and (GB-30998) from the National Science Foundation.

REFERENCES

1 Strotman, H., Weninger, R. and Von Gösseln, C. (1972) *FEBS Lett.* 21, 289–292
2 Mantai, K. E. (1970) *Plant Physiol.* 45, 563–566
3 Selman, B. R. and Bannister, T. T. (1971) *Biochim. Biophys. Acta* 253, 428–436
4 Hoch, G. (1972) in *Methods in Enzymology* (San Pietro, A., ed.), p. 297
5 Itoh, M., Yamashita, K., Nishi, T., Konishi, K. and Shibata, K. (1969) *Biochim. Biophys. Acta* 180, 509-519
6 Cheniae, G. M. and Martin, I. F. (1971) *Plant Physiol.* 47, 568–575
7 Bamberger, E. S. and Park, R. B. (1966) *Plant Physiol.* 41, 1591–1600
8 Izawa, S. and Good, N.E. (1966) *Plant Physiol.* 41, 544–552
9 Greenblatt, C. L., Olson, R. A. and Engel, E. K. (1960) *Biophys. Biochem. Cytol.* 7, 235–238
10 Lien, S. and Bannister, T. T. (1971) *Biochim. Biophys. Acta* 245, 465–481
11 Rurainski, H. (1971) Ph.D. Thesis, University of Rochester
12 Hiyama, T. and Ke, B. (1972) *Biochim. Biophys Acta* 267, 160–171
13 Heath, R. L. and Hind, G. (1969) *Biochim. Biophys. Acta* 189, 222–233
14 Yamashita, T. and Butler, W. L. (1968) *Plant Physiol.* 43, 1978–1986
15 Dilley, R. A. (1970) *Arch. Biochem. Biophys.* 137, 270
16 Arntzen, C, J., Dilley, R. A. and Crane, F. L. (1969) *J. Cell Biol.* 43, 16–31
17 Cheniae, G. M. and Martin, I. F. (1968) *Biochim. Biophys. Acta* 153, 819–837
18 Anderson, J. M. and Vernon, L. P. (1967) *Biochim. Biophys. Acta* 143, 363–376
19 McCarty, R. E. and Racker, E. (1967) in *Energy Conversion by the Photosynthetic Apparatus*, Brookhaven Natl. Lab. No. 19, pp. 202–214, Upton, N.Y.
20 Lynn, W. S. and Straub, K. D. (1969) *Biochemistry* 8, 4789–4793
21 Howell, S. H. and Moudrianakis, E. N. (1967) *Proc. Natl. Acad. Sci. U.S.* 58, 1261–1268
22 Homann, P. H. (1967) *Plant Physiol.* 42, 997–1007
23 Murakami, S. (1968) in *Comp. Biochem. and Biophys. of Photosynthesis* (Shibata, S., Takamiya, A., Jagendorf, A. and Fuller, R., eds), pp. 82–88, Univ. Park Press, State College, Pa.
24 Dilley, R. A., Peters, G. A. and Shaw, E. R. (1972) *J. Membrane Biol.* 8, 163
25 Hind, G. and Jagendorf, A. T. (1963) *Proc. Natl. Acad. Sci. U.S.* 49, 715–722
26 Neumann, J. and Jagendorf, A. T. (1964) *Arch. Biochem. Biophys.* 107, 109
27 Karlish, S. J. D. and Avron, M. (1968) *Biochim. Biophys. Acta* 153, 868–877
28 Izawa, S., Hind, G. (1967) *Biochim. Biophys. Acta* 143, 377–390
29 Gross, E. L. and Packer, L. (1967) *Arch. Biochem. Biophys.* 121, 779

30 Arntzen, C. J., Dilley, R. A. and Crane, F. L. (1969) *J. Cell Biol.* 43, 16
31 Arnon, D. I., Chain, R. K., McSwain, B. D., Tsujimoto, H. Y. and Knoff, D. B. (1970) *Proc. Natl. Acad. Sci. U.S.* 67, 1404
32 Sane, P. V., Goodchild, D. J. and Park, R. B. (1970) *Biochim. Biophys. Acta* 216, 162–178
33 Rurainski, H. J., Randles, J. and Hoch, G. E. (1971) *FEBS Lett.* 13, 98