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TRYPSIN INHIBITION OF PHOTOSYNTHETIC PYRIDINE NUCLEOTIDE REDUCTION

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SUMMARY

1. Photosynthetic pyridine nucleotide reduction is rapidly inhibited by trypsin, the extent of which depends on (i) the order of addition of components to reaction mixtures and (ii) the amount of trypsin added. The inhibited rate appears to be a steady-state rate and is a function of the concentration of trypsin.

2. A partial restoration of the rapidly inhibited rate of $NADP^+$ reduction can be achieved by subsequent addition of trypsin inhibitor.

3. Rapid inhibition by trypsin is not manifested in either methylviologen-catalyzed oxygen consumption or ferredoxin-dependent cytochrome *c* photoreduction. Nor is there any effect of trypsin on ferredoxin–$NADP^+$ reductase-catalyzed diaphorase reactions.

4. It is suggested that trypsin binds rapidly to the chloroplast membrane and inhibits electron flow from the primary acceptor of Photosystem I to ferredoxin–$NADP^+$ reductase.

INTRODUCTION

Trypsin treatment of spinach chloroplasts has been shown to have several effects on photosynthetic electron transport. From studies of the kinetics of inhibition of 2,6-dichlorophenolindophenol (DCIP) reduction, using either water or the System II donor 1,5-diphenylcarbazide (DPC) as the electron source, we previously concluded that trypsin inhibits electron transport at two sites, both on the oxidizing side of System II [1]. In addition, Mantai [2] has shown that mild trypsin treatment accelerates the basal rate of non-cyclic electron transport and he concluded that trypsin uncouples phosphorylation from electron transport. We have measured the P/e_2 ratio for non-cyclic electron transport and confirmed Mantai's conclusions [3]. Strotman

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol; DABS, diazoniumbenzene sulfonic acid; DSPD, disalicylidenepropanediamine.

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et al. [4] demonstrated that trypsin also inhibits cyclic phosphorylation while we have shown that trypsin does not inhibit phenazine methosulfate (PMS) catalyzed cyclic P_{700} flux [3]. From these results, we concluded that trypsin also uncouples cyclic phosphorylation from electron transport [3].

Evidence has been presented that trypsin does not inhibit electron transport activity of System I measured as either ascorbate plus DCIP to methylviologen (MV) [1] or P_{700} flux supported by PMS [3]. From these findings we would predict that trypsin should inhibit $NADP^+$ reduction supported by water, the inhibition being at System II, but not that supported by ascorbate plus DCIP. Mantai [2] reported that ascorbate plus DCIP supported $NADP^+$ reduction is insensitive to trypsin; however, preliminary experiments of ours showed that under at least some conditions there is a strong inhibition by trypsin in the System I donor reaction, and moreover, trypsin may inhibit $NADP^+$ reduction in the open system in a manner not entirely accounted for by inactivation in System II.

Experiments reported in this paper will show that $NADP^+$ reduction is inhibited in System I, apparently as a result of trypsin complexing with chloroplasts and inhibiting reduction of bound ferredoxin–$NADP^+$ reductase by ferredoxin.

METHODS

Chloroplasts were prepared from spinach in buffer containing 10 mM Tris–HCl (pH 7.6), 10 mM NaCl, and 0.4 M sucrose as previously described [1]. $NADP^+$ and cytochrome *c* (Grade III, Sigma Chemical Company) reduction were measured spectrophotometrically at 340 and 550 nm, respectively, in the cross-beam spectrophotometer described by Selman and Bannister [1]. Methylviologen-catalyzed oxygen consumption was measured polarographically with a Clark-type electrode (YSI Model 5331).

Crude ferredoxin was isolated from spinach by the following procedure. Approximately 0.5 kg of spinach were ground in 1 l of buffer containing 0.4 M sucrose, 0.02 M Tricine (pH 7.5), and 0.02 M NaCl in a Waring blender for 20 s. The homogenate was filtered through cheese cloth and centrifuged at $4000 \times g$ to sediment the chloroplasts. The chloroplasts were resuspended in buffer and the supernatant recycled until 10 kg of spinach were processed. The crude chloroplasts were fractionated with pre-chilled (−15 °C) acetone (10 : 1, v/v) and the pellet air dried at 0–4 °C. The pellet was extracted with 0.02 M Tricine (pH 7.5) and 0.02 M NaCl buffer and the extract was (i) dialyzed against Tricine buffer for 24 h and (ii) adsorbed onto a DEAE-cellulose column. Crude ferredoxin was eluted from the column in Tricine buffer containing 0.3 M NaCl and was used without further purification. The crude preparation catalyzed both the ferredoxin dependent photoreduction of $NADP^+$ and the dark reduction of cytochrome *c* with NADPH, a reaction which requires both ferredoxin and ferredoxin–$NADP^+$ reductase [5].

Ferredoxin–$NADP^+$ reductase was purified by the method of Shin et al. [6].

For kinetic studies of trypsin inhibition of $NADP^+$ reduction, trypsin treatment was performed as previously described at a trypsin to chlorophyll ratio of 0.13 [1].

RESULTS

Trypsin inhibition of Photosystem I and II

As will be seen shortly, the order of addition of components is important in determining whether or not inhibition occurs. In our previous experiments, the usual practice was to incubate chloroplasts with trypsin (for samples) or without trypsin (for controls); at intervals during incubation, aliquots were removed, trypsin inhibitor added, and then electron transport and uncoupling reagents. Fig. 1 shows the inhibition of the reaction water to $NADP^+$ by trypsin, with samples prepared in this customary manner. The point on the graph for the sample treated with trypsin for zero time was obtained by taking an aliquot from the chloroplast suspension incubated without trypsin, adding trypsin inhibitor, then trypsin, and finally $NADP^+$, ferredoxin and uncoupler.

Fig. 1 shows a steadily increasing inhibition by trypsin (half-time approximately 1.3 min) which is reasonably identified with inactivation of System II [1]. Additionally, it is clear from (i) the inhibition actually manifested by the zero time sample, and (ii) the apparent inhibition at zero time indicated by extrapolating the decay curve, that a kinetically different fast inhibition occurs in the first seconds following the exposure of chloroplasts to trypsin.

The fast inhibition can also be manifested in the time course of the reaction ascorbate plus DCIP to $NADP^+$ (Fig. 2). When trypsin was added (Curve A), the rate of reaction decreased suddenly (within 10 s) to a new lower steady-state value. A

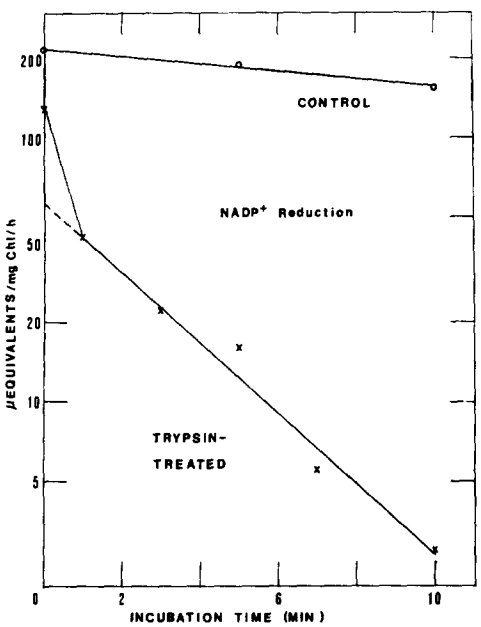


Fig. 1. Kinetics of trypsin inhibition of $NADP^+$ reduction. Reaction mixtures contained in 3.0 ml, chloroplasts at 10 μg chlorophyll/ml, 33 mM methylamine · HCl, 5 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.6), 10 mM NaCl, 1.0 mM $NADP^+$, and 33 μg/ml ferredoxin (Grade III, Sigma Chemical Company). Incident illumination about $5 \cdot 10^5$ ergs · $cm^{-2} \cdot s^{-1}$.

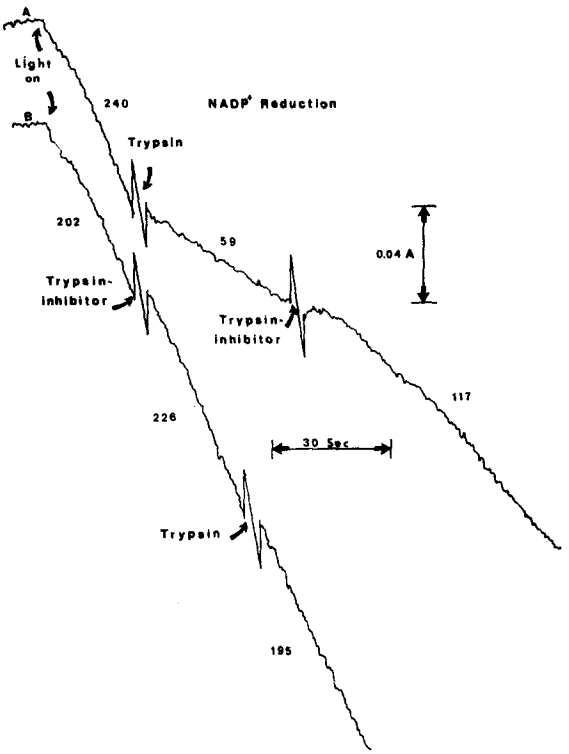


Fig. 2. Trypsin inhibition of $NADP^+$ reduction by ascorbate plus DCIP. Reaction mixtures contained in 3.0 ml, chloroplasts at 20 μg chlorophyll/ml, 10 mM Tris–HCl (pH 7.6), 10 mM NaCl, 33 mM methylamine · HCl, 0.5 mM $NADP^+$, 6.6 μM DCMU, 1.0 mM ascorbate, 0.1 mM DCIP, and a saturating amount of crude ferredoxin. Where indicated 6.6 μg/ml trypsin or 13.2 μg/ml trypsin inhibitor was added. Numbers refer to the rate of $NADP^+$ reduction in μequiv./mg chlorophyll per h.

subsequent addition of trypsin inhibitor caused a gradual recovery (over about 30 s) of a part of the original activity. These and other experiments showed that there was never any gradually increasing inhibition of the donor reaction with time after addition of trypsin. The rate after reversal ranged from 120 to 200% of the inhibited rate in samples prepared by adding chloroplasts, crude ferredoxin, uncoupler and then trypsin immediately before illumination. The cause of this wide variation in results is not known.

In both Figs 1 and 2, it is evident that the fast inhibition causes a rapid loss of a part of the original activity, but does not continue to completion as one would expect if the inhibition were due to a progressive hydrolysis by dissolved trypsin. Rather the observed properties suggest that fast inhibition is due to the quick formation of a stable complex of trypsin with another component necessary for activity. Curve B in Fig. 2 shows that, when trypsin inhibitor is present, trypsin addition no longer inhibited the reaction. An obvious explanation would be that trypsin inhibitor complexes trypsin rapidly and forms a more stable complex than the component required for activity. It will be shown below that the presence of trypsin inhibitor does not always completely protect against trypsin inhibition.

Remarks on measurement of fast inhibition

Since trypsin causes only fast inhibition and not any slow progressive inhibition of the Photosystem I reaction, ascorbate plus DCIP to $NADP^+$, there is no difficulty in measuring the extent of fast inhibition. On the other hand, in the reaction water to $NADP^+$, the progressive inhibition of System II might be expected to interfere with accurate measurement of the fast inhibition. In actuality, as Fig. 2 indicated, it was generally possible to estimate reaction rates fairly precisely from as little as a 15–20-s interval of the strip chart. As Fig. 1 shows, this is too short a period for development of a significant inhibition in System II. Fast inhibition is fully developed in a few seconds and it was therefore generally possible to determine the extent of fast inhibition in the reaction water to $NADP^+$ even in the absence of trypsin inhibitor.

Effects of order of addition; evidence of protection by crude ferredoxin

It was found that the extent of fast inhibition changed when the order of adding components was varied. The effects were studied with samples prepared by placing diluted chloroplasts in the reaction vessel and then adding in varying order, trypsin, trypsin inhibitor, or crude ferredoxin. A mixture of all the other reaction components [i.e., ferredoxin with some accompanying reductase, $NADP^+$, and methylamine for the reaction water to $NADP^+$ or ferredoxin (with reductase), $NADP^+$, ascorbate, DCIP, methylamine, and DCMU for the donor reaction] were added with the crude ferredoxin. The volumes of the additions of trypsin, trypsin inhibitor, and crude ferredoxin were all negligible compared with the volume of the diluted chloroplasts.

Tables I and II, and the first columns of Table III indicate several effects. First of all, comparison of the extent of fast inhibition of Samples 4 and 5 in Table I and Samples 3 and 4 in Table II shows that the inhibition is always less when crude ferredoxin is added before trypsin and trypsin inhibitor; evidently a component of crude ferredoxin partially protects against inhibition. Secondly, as shown by the

TABLE I

THE EFFECT OF THE ORDER OF ADDITION ON TRYPSIN INHIBITION OF $NADP^+$ REDUCTION WITH WATER AS DONOR

Reaction mixtures for $NADP^+$ reduction contained in 3.0 ml, chloroplasts at 20 μg chlorophyll/ml, 33 mM methylamine · HCl, 10 mM Tris–HCl (pH 7.6), 10 mM NaCl, 0.5 mM $NADP^+$, a saturating amount of crude ferredoxin and (where indicated) 6.6 μg/ml trypsin and 13.2 μg/ml trypsin inhibitor. Arrows indicate order of additions. Ferredoxin, reductase, methylamine · HCl and $NADP^+$ were all added simultaneously. Incident illumination about $5 \cdot 10^5$ ergs · cm^{-2} · s^{-1}.

Reaction: $H_2O \rightarrow NADP^+$.

Order of addition	μequiv./mg chlorophyll per h	% Control
(1) chloroplast → ferredoxin	375	100
(2) chloroplast → trypsin inhibitor → ferredoxin	344	92
(3) chloroplast → ferredoxin → trypsin inhibitor	356	95
(4) chloroplast → ferredoxin → trypsin inhibitor → trypsin	350	93
(5) chloroplast → trypsin inhibitor → trypsin → ferredoxin	274	73

TABLE II

THE EFFECT OF THE ORDER OF ADDITION ON TRYPSIN INHIBITION OF $NADP^+$ REDUCTION WITH ASCORBATE PLUS DCIP AS DONOR

Reaction mixtures for $NADP^+$ reduction contained in 3.0 ml, chloroplasts at 20 μg chlorophyll/ml, 33 mM methylamine · HCl, 10 mM Tris–HCl (pH 7.6), 10 mM NaCl, 0.5 mM $NADP^+$, a saturating amount of crude ferredoxin, 6.6 μM DCMU, 1 mM ascorbate, 0.1 mM DCIP, and (where indicated) 6.6 μg/ml trypsin and 13.2 μg/ml trypsin inhibitor. Ferredoxin, reductase, methylamine · HCl, $NADP^+$, DCMU, ascorbate, and DCIP were all added simultaneously.

Reaction: ascorbate/DCIP → $NADP^+$.

Order of addition	μequiv./mg chlorophyll per h	% Control
(1) chloroplasts → ferredoxin	208	100
(2) chloroplasts → trypsin inhibitor → ferredoxin	205	98
(3) chloroplasts → ferredoxin → trypsin inhibitor → trypsin	178	86
(4) chloroplasts → trypsin inhibitor → trypsin → ferredoxin	125	60
(5) chloroplasts → ferredoxin → trypsin	0	0

TABLE III

EFFECT OF TRYPSIN ON SEVERAL PHOTOREACTIONS

Conditions were chosen to maximize the possibility for trypsin inhibition. Reaction mixtures for $NADP^+$ reduction from water as in Table I and from ascorbate plus DCIP as in Table II. Reaction mixtures for methylviologen-catalyzed oxygen consumption contained in 3.0 ml, chloroplasts at 10 μg chlorophyll/ml, 1.0 mM KCN, 6.6 μM DCMU, 1.0 mM ascorbate, 0.1 mM DCIP, 10 mM Tris–HCl (pH 7.6), 10 mM NaCl, 33 mM methylamine · HCl, and 0.5 mM methylviologen. Samples were assayed for oxygen consumption with a Clark electrode. Reaction mixtures for cytochrome *c* reduction contained in 3.0 ml, chloroplasts at 10 μg chlorophyll/ml, 10 mM Tris–HCl (pH 7.6), 10 mM NaCl, 33 mM methylamine · HCl, 0.5 mM cytochrome *c*, and a saturating amount of crude ferredoxin. An extinction coefficient of 19 $cm^{-1} \cdot mM^{-1}$ at 550 nm was assumed. Trypsin inhibitor (13.2 μg/ml) (Row 3) or trypsin inhibitor plus trypsin (6.6 μg/ml) (Row 4) was added to chloroplasts before the addition of other electron transport reagents. When trypsin was added alone (Row 5), the addition to the complete reaction mixture was immediately prior to illumination. Control rates: 300, 111, 250 and 1011 μequiv./mg chlorophyll per h for H_2O → $NADP^+$, Asc/DCIP → $NADP^+$, H_2O → cytochrome *c* and Asc/DCIP → methylviologen, respectively.

Reaction	Percent of control			
	H_2O → $NADP^+$	Asc/DCIP → $NADP^+$	H_2O → cytochrome *c*	Asc/DCIP → methylviologen
Trypsin/chlorophyll ratio	0.33	0.33	0.66	0.66
Control	100	100	100	100
+trypsin inhibitor	91	88	–	100
+trypsin, +trypsin inhibitor	54	55	100	94
+trypsin	35	31	100	87

TABLE IV

PROTECTION AGAINST TRYPSIN INHIBITION BY TRYPSIN INHIBITOR

Reaction mixtures as in Table I. Trypsin inhibitor (as indicated) and chloroplasts were mixed prior to the addition of trypsin (6.6 μg/ml) in Column 3 and trypsin and trypsin inhibitor were mixed prior to the addition of chloroplasts in Column 4. Note that methylamine · HCl and $NADP^+$ were added simultaneously with ferredoxin after the addition of trypsin. Numbers in parentheses are percent control.

Reaction: $H_2O \rightarrow NADP^+$.

Trypsin inhibitor (μg/ml)	$NADP^+$ reduction (μequiv./mg chlorophyll per h)		
	Control	Trypsin added to chloroplasts plus trypsin inhibitor	Chloroplasts added to trypsin plus trypsin inhibitor
0	410	75 (18)	–
3.3	371	121 (33)	363 (98)
6.6	350	162 (46)	376 (107)
13.2	384	232 (60)	330 (86)
33.0	339	239 (71)	287 (85)
66.0	311	269 (86)	280 (90)

total inhibition of Sample 5 in Table II, fast inhibition can go to completion even though in this case crude ferredoxin was present. In Table III, Columns 1 and 2 show that trypsin added after crude ferredoxin gave a large but not total inhibition. Evidently trypsin has the potential for totally inhibiting $NADP^+$ reduction; the extent of fast inhibition actually realized depends on the relative concentrations of trypsin and crude ferredoxin. Thirdly, trypsin inhibitor alone at the concentrations used can give a slight inhibition; this is shown by Sample 2 in Table I, by Sample 3 in Table II, and again in Table III. At higher concentrations of trypsin inhibitor, the extent of inhibition increases (see Table IV). The slight inhibition observed with normal concentrations is therefore not due to complexing with a significant fraction of chloroplast binding sites. Stated differently, with normal concentrations of trypsin inhibitor, most chloroplast sites remain unbound, and the protective action of trypsin inhibitor is not due to blocking sites of trypsin binding. Fourthly, Samples 3, 4 and 5 in Table II show that crude ferredoxin could not protect against trypsin alone, but could partially protect when trypsin inhibitor was also present.

Extent of fast inhibition: effect of trypsin concentration in the absence of trypsin inhibitor

Some characteristics of the binding of trypsin to chloroplasts were established by investigating the effect of trypsin concentration on the extent of fast inhibition, in the absence of trypsin inhibitor. The reaction studied was water to $NADP^+$. As stated above, it is possible to measure the rate after fast inhibition is fully developed but before any appreciable inhibition of System II occurs. Results are shown in Fig. 3. It is evident that there is a small fraction (about 10–15%) of the total control rate that is insensitive to trypsin inhibition. This residual activity might be due to (i) a residual activity of blocked sites, (ii) inaccessibility of a small fraction of the sites, or

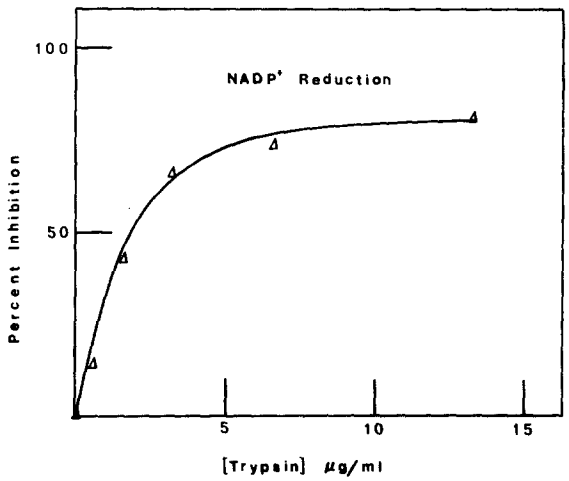


Fig. 3. Concentration curve for trypsin inhibition of $NADP^+$ reduction. Reaction mixtures contained in 3.0 ml, chloroplasts at 20 µg chlorophyll/ml, 10 mM Tris–HCl (pH 7.6), 10 mM NaCl, 33 mM methylamine · HCl, 0.5 mM $NADP^+$, and 33 µg/ml ferredoxin (Grade III, Sigma Chemical Company). Trypsin was added during illumination to the complete reaction mixture. Control rate was 286 µequiv./mg chlorophyll per h.

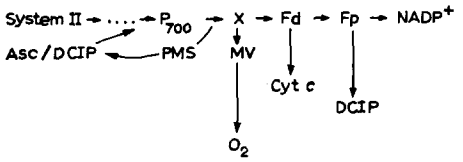
(iii) some pyridine nucleotide reduction by soluble rather than chloroplast-bound ferredoxin–$NADP^+$ reductase (it will be shown later that soluble terminal electron transport reactions with soluble reductase are insensitive to trypsin). Because (i) trypsin inhibition of $NADP^+$ reduction can be partially reversed by adding trypsin inhibitor and (ii) the inhibited rate is a steady-state rate and is a function of the amount of trypsin added, not the time of incubation, fast inhibition may be due to binding of trypsin to the chloroplast and not to trypsin hydrolysis. This complex apparently dissociates when trypsin inhibitor is added.

Extent of fast inhibition: effect of concentration of trypsin inhibitor

To determine whether or not both trypsin and the complex of trypsin and trypsin inhibitor are active in blocking pyridine nucleotide reduction, the concentration of trypsin inhibitor and the order of addition of trypsin were varied. Table IV (Column 3) shows the results for an experiment where trypsin inhibitor and chloroplasts were mixed prior to the addition of trypsin and crude ferredoxin. Apparently, very high concentrations of trypsin inhibitor will protect chloroplasts from trypsin inhibition (trypsin inhibitor itself being a weak inhibitor at high concentrations). If however, trypsin inhibitor and trypsin are mixed prior to the addition of chloroplasts and crude ferredoxin (Table IV, Column 4), there is no inhibition of $NADP^+$ reduction even at the lowest concentration of trypsin inhibitor tested. This experiment indicates the following: (i) at normal concentrations, trypsin inhibitor does not complex with a significant fraction of chloroplast binding sites, (ii) even at normal concentrations, trypsin inhibitor will completely complex trypsin and the resulting complex is non-inhibitory, and (iii) only free trypsin can act on chloroplasts giving inhibition.

Location of the System I binding site for trypsin

It is believed electron transport reactions in System I can be represented schematically as follows [5, 7, 8]:



Fd, ferredoxin; Fp, ferredoxin-$NADP^+$ reductase; MV, methylviologen; Cyt *c*, cytochrome *c*.

It has been shown that trypsin has no effect on methylviologen reduction (ref. 1, also Table III), nor on cyclic electron transport with PMS [3]. It therefore seems probable that the site of trypsin binding in System I lies between the primary acceptor (X) and $NADP^+$.

In order to determine if trypsin binds ferredoxin or perhaps the site on the chloroplast membrane which normally binds ferredoxin, the effect of trypsin on the Hill reaction with cytochrome *c* was investigated. This reaction requires the primary System I acceptor and ferredoxin, but not ferredoxin–$NADP^+$ reductase [5]. There is no inhibition by trypsin either with saturating levels (Table III) or with rate-limiting levels (Table V) of ferredoxin. Evidently, trypsin does not interfere with ferredoxin functions in this reaction.

The insensitivity of cytochrome *c* reduction to trypsin suggests that the site of trypsin inhibition of $NADP^+$ reduction occurs after the photoreduction of ferredoxin and that trypsin does not directly interact with ferredoxin. If this is correct, then trypsin inhibition of $NADP^+$ reduction should be independent of the ferredoxin concentration when trypsin is added before ferredoxin. That this is indeed the case is shown in Table VI. The percent inhibition by trypsin remains constant over a 20-fold range in ferredoxin concentration.

TABLE V

EFFECT OF FERREDOXIN CONCENTRATION ON CYTOCHROME *c* REDUCTION IN THE PRESENCE OF TRYPSIN

Reaction mixtures for cytochrome *c* reduction as in Table III. Crude ferredoxin (μl of stock preparation) and 6.6 μg/ml trypsin were added immediately before illumination.
Order of addition: chloroplasts → cytochrome *c* → ferredoxin → trypsin; Reaction: H_2O → cytochrome *c*.

Relative ferredoxin concn	μequiv./mg chlorophyll per h		% Control
	−Trypsin	+Trypsin	
0	24	31	129
2	50	60	120
5	76	76	100
50	125	123	99

TABLE VI

EFFECT OF FERREDOXIN ON TRYPSIN INHIBITION OF $NADP^+$ REDUCTION

Reaction mixtures as in Table I contained trypsin inhibitor (13.2 μg/ml), trypsin (6.6 μg/ml), and crude ferredoxin as indicated (μl stock preparation).

Order of addition: chloroplasts → trypsin inhibitor → trypsin → ferredoxin; Reaction: H_2O → $NADP^+$.

Relative ferredoxin concn	$NADP^+$ reduction (μequiv./mg chlorophyll per h)		% Control
	Control	plus trypsin plus trypsin inhibitor	
5	34	20	59
10	77	40	52
20	174	109	63
50	347	218	63
100	357	210	59

After ferredoxin, the only other known component in $NADP^+$ reduction is ferredoxin–$NADP^+$ reductase. An inhibitory interaction between this enzyme and trypsin therefore appeared probable. To test for such an interaction, the effect of trypsin on two diaphorase reactions was investigated. In one, the reduction of DCIP by NADPH, only the reductase is required [6]; in the other, the reduction of cytochrome *c* by NADPH, both reductase and ferredoxin are necessary [5].

The effect of trypsin was first examined when these two reactions were carried out with dissolved enzymes. For this, samples contained oxidant (DCIP or cytochrome *c*), NADPH, and enzyme(s), but no chloroplasts. The enzyme preparations available included highly purified reductase (which catalyzed the DCIP but not the cytochrome *c* reaction) and a crude ferredoxin preparation which contained ferredoxin and substantial reductase activity as well. When the cytochrome *c* reaction was carried out with a rate-limiting amount of crude ferredoxin, addition of the purified reductase did not stimulate the reaction. From this it appears that the crude preparation included an even higher reductase than ferredoxin activity and therefore that the cytochrome *c* reaction was always ferredoxin-limited. Surprisingly, as Table VII shows, trypsin was without effect on either reaction.

It was also possible to carry out the NADPH to DCIP and cytochrome *c* reactions in the presence of chloroplasts (which contain bound reductase). In these reactions, only ferredoxin needed to be added for cytochrome *c* reduction. Trypsin again had no effect.

These negative results leave the site of trypsin uncertain. One way the results might be rationalized is to suppose that trypsin binds to a chloroplast component (not ferredoxin or reductase), and that the resulting complex then interferes with electron transport between ferredoxin and the reductase. In this way, one can account for the absence of any effect on (i) the NADPH to DCIP reaction (which does not depend on transfer from reductase to ferredoxin), (ii) the soluble NADPH to cytochrome *c* reaction (the trypsin binding component is not present), and (iii) the Hill reaction with cytochrome *c* (again transfer from ferredoxin to reductase is not involved).

TABLE VII

EFFECT OF TRYPSIN ON DARK REACTIONS CATALYZED BY CRUDE FERREDOXIN AND FERREDOXIN–$NADP^+$ REDUCTASE

Reaction mixtures for DCIP reduction contained in 3.0 ml, 80 μM DCIP, 200 μM NADPH, 5 μl stock preparation of ferredoxin–$NADP^+$ reductase, 10 mM Tris–HCl (pH 7.6), and 10 mM NaCl. The reaction was initiated by the addition of the reductase and was monitored at 590 nm in a Perkin–Elmer (124) recording spectrophotometer at room temperature. Reaction mixtures for cytochrome *c* reduction contained in 3.0 ml, 100 μM cytochrome *c*, 50 μl stock preparation of crude ferredoxin, 10 mM Tris–HCl (pH 7.6), and 10 mM NaCl. The reaction was initiated by the addition of ferredoxin and was monitored at 550 nm at room temperature. Trypsin and trypsin inhibitor were added to a final concentration of 6.6 and 13.2 μg/ml, respectively.

	Reaction (% of control)	
	NADPH → DCIP*	NADPH → cytochrome *c***
Control	100	100
+trypsin inhibitor	105	–
+trypsin		
+trypsin inhibitor	108	100
+trypsin	100	100

* Control rate 0.327 $A_{590\ nm}$/min per 5 μl ferredoxin–$NADP^+$ reductase stock.
** Control rate 0.148 $A_{550\ nm}$/min per 50 μl stock ferredoxin.

DISCUSSION

Photoreduction of $NADP^+$ is believed to involve complex formation between ferredoxin and ferredoxin–$NADP^+$ reductase [9]. Several compounds, for example pyrophosphate [5] and DSPD [10], as well as high ionic strength [11], have been shown to prevent the formation of the complex with isolated enzymes and inhibit photoreduction of $NADP^+$. Trypsin also appears to inhibit terminal electron transport, but unlike pyrophosphate, DSPD, and DABS [12] which inhibit all ferredoxin mediated reactions, trypsin has no inhibitory effect on cytochrome *c* photoreduction. Nor does trypsin inhibit the dark reduction of cytochrome *c* by NADPH, an effect not very well understood.

Clearly, the interaction between trypsin and chloroplasts resulting in inhibition of $NADP^+$ reduction is complex. One explanation might be that trypsin forms three reversible complexes: one with chloroplasts, which prevents the reduction of $NADP^+$, another with trypsin inhibitor which is inactive as an inhibitor of $NADP^+$ reduction, and a third with a component of crude ferredoxin. Inhibition then would be determined by the amount of trypsin partitioned between chloroplasts, trypsin inhibitor, and crude ferredoxin. According to this model, the protecting effect of crude ferredoxin would be the result of some component of the preparation (either ferredoxin, reductase, or some other unknown component) complexing with trypsin. In order for the crude preparation to have a protecting effect against fast inhibition, the trypsin–unknown component complex must be stable, i.e., both the rate constant for dissociation of the complex and the time constant for complex formation must be small. Thus, trypsin, added to a reaction mixture containing both crude ferredoxin and trypsin inhibitor, would rapidly be tied up in two stable complexes (one with trypsin

inhibitor and the other with the unknown component).

This model, however, leaves unexplained several important observations:

(i) Table IV clearly shows that when trypsin and trypsin inhibitor are mixed together prior to the addition of chloroplasts there is no inhibition of $NADP^+$ reduction. Similar experiments have shown this result to be independent of time. Yet, the addition of trypsin inhibitor (at five times the concentration needed to prevent inhibition, see Table IV) in experiments similar to the one shown in Fig. 2A never restores the inhibited rate to more than about 50% of the uninhibited control rate. Certainly if the equilibrium constants (between chloroplasts and trypsin inhibitor) differ by a large factor trypsin inhibitor should complex all of the trypsin and reverse inhibition totally.

(ii) Tables I and II show that the order of addition of electron transport components determines the amount of inhibition. This would not be the case if only chloroplasts, trypsin inhibitor, and crude ferredoxin were competing for trypsin. Since the rate-limiting step would be the dissociation of the trypsin–chloroplast complex, order of addition effects should not last longer than about 20 s (the time to completely dissociate the trypsin chloroplast complex and form the more stable trypsin–trypsin inhibitor and trypsin–unknown component complexes). In addition, the concentration of crude ferredoxin has no effect on the percent inhibition by trypsin if added to the reaction last (Table VI).

(iii) The fast inhibition of $NADP^+$ reduction cannot be entirely relieved by washing chloroplasts in isolation media.

One way to explain order of addition and partial reversal would be to suppose that trypsin quickly complexes chloroplasts (the complex being photosynthetically inactive, but capable of regenerating active chloroplasts on dissociation), and in addition, that a slow hydrolysis occurs within the complex which leads to permanent inactivation not reversed by dissociation.

According to the model composed of the equilibria and the additional hydrolytic step:

(i) In the absence of trypsin inhibitor, the inhibited steady-state rate would be due to a combination of both trypsin hydrolysis and binding. The reason the rate appears to be steady-state would be due to the low rate constant for hydrolysis.

(ii) Addition of trypsin inhibitor to a reaction mixture containing the trypsin–chloroplast complex would reverse only that portion of the inhibition due to binding. The extent of recovery would decrease with increasing time after addition of trypsin. This could explain the wide variation in results obtained for trypsin inhibitor stimulation.

(iii) The protective effect of crude ferredoxin (when added before trypsin) would be due to binding of some trypsin and a reduction of the amount of trypsin–chloroplast complex formed. Addition of crude ferredoxin after trypsin inhibitor would have no effect on activity recovered.

(iv) Hydrolytic inhibition could not be relieved by washing chloroplasts in buffer after addition of trypsin.

It is surprising that Mantai [2] did not report any inhibition by trypsin on $NADP^+$ reduction in the ascorbate plus DCIP donor system reaction. Without trypsin inhibitor in the incubation mixture, we typically found about 30–50% inhibition at comparable chlorophyll to trypsin ratios. The differences in our results

cannot be attributed to differences in incubation conditions for unlike the sites at System II, which are protected by high sucrose and KCl [3], the System I site is only partially protected (unpublished results). The cause of this discrepancy remains unclear.

Finally, because trypsin is a hydrophilic protein, its effects on Photosystem I are probably limited to components exposed at the external surface of the chloroplast membrane. Our results confirm experiments with antibodies [13, 14] and the water-soluble probe, DABS [12], that the machinery for $NADP^+$ reduction is located externally with respect to the thylakoid membrane. Unlike the reaction with antibodies and DABS, however, we have been unable to find an effect of trypsin on the primary Photosystem I acceptor.

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