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Proteolytic enzymes stimulate both the ATP synthesis and hydrolysis functions of the chloroplast ATPase complex

C.A. Raines and M.F. Hipkins

Department of Botany, University of Glasgow, Glasgow (U.K.)

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The effects of trypsin and chymotrypsin on phosphorylation and ATP hydrolysis by the ATPase in isolated thylakoid membranes of pea have been compared. Tentoxin-sensitive hydrolysis stimulated by both treatments in the light led to reverse proton pumping, seen as a quenching of the light-induced fluorescence of 9-aminoacridine, and which was also reflected as an acceleration of the rate of decay of the flash-induced field-indicating absorption change at 515 nm. Treatments in the dark were essentially ineffective. Trypsin treatment in the light (5 min) led to complete inhibition of phosphorylation but only to partial inhibition of light-induced oxygen evolution. However, at short incubation times in the light with trypsin (1 min) there was stimulation both of phosphorylation and of oxygen evolution, whilst no change was observed in the P/O_2 ratio. Similar findings have been reported for dithiothreitol, suggesting that the trypsin-induced stimulation of ATP hydrolysis is not a consequence of uncoupling but results from an alteration in the catalytic state of the ATPase complex. In contrast, chymotrypsin inhibited both O_2 evolution and phosphorylation: no stimulation in either of these parameters was seen under the conditions investigated.

Introduction

The ATPase complex catalyses both the synthesis and hydrolysis of ATP. In the chloroplast this enzyme is under strict regulatory control and requires activation to reveal any catalytic activity [1,2]. Activation of the complex is believed to be mediated by an electrochemical gradient of protons $\Delta\mu_{H^+}$ [1–3] which is generated by light-induced electron transport. The mechanism of activation is unknown but light-induced conformational changes [4,5] which aid the release of bound nucleotides [6–9] have been implicated.

Although $\Delta\mu_{H^+}$ (generated in the light) activates ATP synthesis in isolated thylakoid membranes, this alone is not sufficient to stimulate ATP hydrolysis. The hydrolytic activity of the ATPase is only revealed when, in addition to $\Delta\mu_{H^+}$ activation, treatment is given in the presence of a thiol compound [10]. The hydrolysis thus produced is dependent on the presence of Mg^{2+} and remained stable in the dark for several minutes. A number of other treatments has subsequently been shown to stimulate ATP hydrolysis in isolated thylakoid membranes: heat [11]; methanol [12,13] and trypsin [14]. ATP hydrolysis stimulated by the thiol compound DTT and by trypsin appeared to have

Abbreviations: $\Delta\mu_{H^+}$, ΔpH difference in the electrochemical potential of protons across the thylakoid membrane; CF_1, soluble portion of the membrane-bound ATPase complex; P/O_2, number of moles of ATP synthesized per mole of O_2 transported from H_2O to methyl viologen; DTT, dithiothreitol; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; Chl, chlorophyll.

Correspondence: C.A. Raines, (present adress:) Plant Breeding Institute, Maris Lane, Trumpington, Cambridge CB2 2LQ, U.K.

similar characteristics, with the exception that trypsin had a slight uncoupling effect [3]. It was suggested by Moroney and McCarty [15] that trypsin-stimulated ATP hydrolysis was linked to the partial uncoupling of ATP synthesis. Both the trypsin- and DTT-stimulated hydrolysis depend on the presence of light during the treatment. It has been suggested that a sulphydryl group on the γ subunit, exposed on activation of the CF_1 complex, is altered by DTT and trypsin treatments thereby shifting the catalytic function of the ATPase complex into the hydrolytic mode [16,17]. The $\Delta\mu_{H^+}$ and the thiol regulatory controls of the ATPase complex were initially observed in vitro but a similar in vivo system involving thioredoxin, a thiol protein with redox reactive groups, was identified by Mills and Hind [18]. The effect of thiol modulation on the rate of ATP synthesis was unknown, but recently it has been shown that both in vitro and in vivo thiol modulation resulted in a stimulation of ATP synthesis and in a lowering of the pH range over which CF_1 is active [2,19,20].

Given the similarities between the effects of DTT and trypsin on ATP hydrolysis, the effect of trypsin on ATP synthesis was studied. In addition a comparative study was made between the effects of trypsin and of chymotrypsin on the functions of the ATPase complex. To address these questions use has been made of the flash-induced field indicating absorption change at 515 nm (ΔA_{515}) [21], the decay rate of which has been shown to be sensitive to the activation of the chloroplast ATPase [22–25]. The feasibility of using trypsin and chymotrypsin to investigate further the structure and functional relationships in the CF_1 complex is discussed.

Materials and Methods

Chloroplasts were isolated from peas (*Pisum sativum* var. Little Marvel) which had been grown for 10–14 days in vermiculite at 23° C and a light intensity of 15 $W \cdot m^{-2}$ with a photoperiod of 12 h. The plant material (50 g) was homogenised in 200 ml of a medium containing: 0.33 M sorbitol, 50 mM K_2HPO_4, 5 mM $MgCl_2$, 0.1% (w/v) NaCl and 2% (w/v) sodium iso-ascorbate (pH 6.5) [26]. Grinding was at high speed for 5 s in a Polytron homogeniser (Type PT 10-35 Kinematica, Switzerland). The homogenate was filtered first through two and then eight layers of muslin together with a thin layer of cotton wool. The filtrate was centrifuged at $2200 \times g$ for 90 s in a bench centrifuge (MSE-Super Minor) yielding a pellet of chloroplasts. Osmotically shocked chloroplasts were prepared by resuspending the pellet in 2 mM $MgCl_2$, 10 mM KCl and 20 mM Tricine-NaOH (pH 7.4) [27]. The chloroplasts were routinely stored in the dark on ice for a minimum of 1 h prior to use. Chlorophyll was determined spectrophotometrically [28].

Trypsin (Sigma type XIII *N*-tosyl-phenylalanyl chloromethyl ketone treated to exclude chymotrypsin activity to below 0.1%) and chymotrypsin (Sigma type VII *N*-tosyl-lysil-chloromethyl ketone treated to exclude trypsin activity to less than 1%) were purchased from the Sigma Chemical Company, Poole, Dorset. The enzymes were prepared as solutions in 20 mM Tricine buffer (pH 7.4) at 100 times the required concentration. Enzyme treatment was carried out by adding thylakoids to test tubes containing the required concentration of enzyme, incubation was carried out at 23° C after which the reaction was stopped by adding soybean trypsin inhibitor (Sigma Chemical Company) which also totally inhibits chymotrypsin activity as assayed using the method of Schwert and Takenaka [29]. Throughout the experiments in this paper the chlorophyll-to-trypsin ratio was maintained at 5 : 1 whilst that of chymotrypsin was 5 : 3.

Photophosphorylation and ATP-hydrolysis rates were estimated by the pH method of Nishimura et al. [30].

Measurements of the flash-induced absorbance changes were carried out in a laboratory constructed single-beam spectrophotometer in essentially the same manner as described previously [32].

9-Aminoacridine fluorescence was measured in a Laboratory constructed fluorimeter by the method of Schuldiner et al. [33].

Results

The nucleotides ATP and ADP, substrates in the reactions of the ATPase complex, have been

reported to affect the half-time of decay of ΔA_{515} [34,35]. We have shown previously that trypsin, chymotrypsin and dithiothreitol treatment of thylakoid membranes in the light led to an appreciable acceleration in the rate of decay of ΔA_{515} [36]. We concluded that this was due to a stimulation of ATP hydrolysis by these three treatments.

In further support of this conclusion, the results in Fig. 1 indicate that tentoxin, an inhibitor of ATP hydrolysis, prevents the stimulatory effect of ATP on the decay rate of ΔA_{515} of membranes pretreated in the light with DTT, trypsin or chymotrypsin.

The effects of trypsin and chymotrypsin on the rate of ATP hydrolysis are shown in Table I. The data show that trypsin treatment in the light stimulated ATP hydrolysis by a factor of 3.5; in the case of chymotrypsin stimulation by a factor of 2.5 was evident for light-treated samples. No appreciable stimulation of ATP hydrolysis was seen for samples treated in the dark with either trypsin or chymotrypsin. Lynn and Straub [14] have shown that trypsin can stimulate ATP hydrolysis; however, they suggested that chymotrypsin actually inhibited this process. The data presented here (Fig. 1; Table I) strongly suggest that chymotrypsin can stimulate ATP hydrolysis.

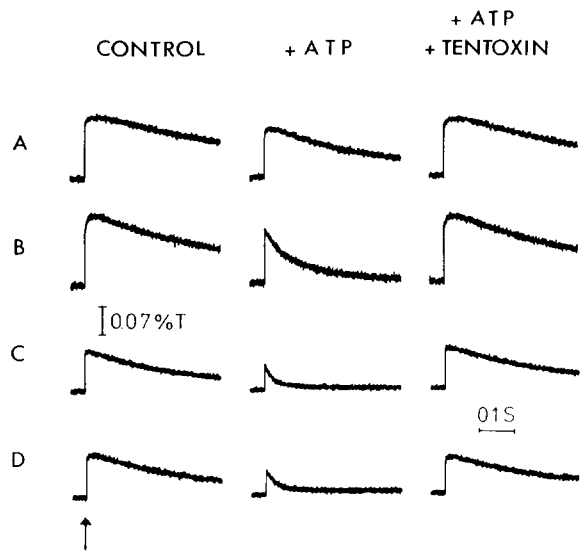

Fig. 1. Effect of ATP on ΔA_{515} of thylakoids pretreated with dithiothreitol, trypsin or chymotrypsin in the presence of tentoxin. Thylakoids with a chlorophyll concentration of 25 $\mu g \cdot ml^{-1}$ were incubated with dithiothreitol (5 mM, 1 min), trypsin (5 $\mu g \cdot ml^{-1}$, 1 min) and chymotrypsin (15 $\mu g \cdot ml^{-1}$, 3 min) in the dark (D) or light (L) in a medium containing Tricine (20 mM), KCl (10 mM), $MgCl_2$ (2 mM) and K_2HPO_4 (2 mM) (pH 7.4). ATP (0.5 mM) and ADP (0.5 mM) were added on completion of incubation. Tentoxin (2 μM) was present during pretreatments where indicated on diagram. (A) Control; (B) dithiothreitol; (C) chymotrpysin; (D) trypsin. The arrow denotes the point at which a flash was fired.

The effect of trypsin and chymotrypsin on the energetic state of the thylakoid membrane was studied using 9-aminoacridine fluorescence. The results show that excitation of the control thylakoids with the actinic light (RL) caused a decrease in 9-aminoacridine fluorescence (Fig. 2A): this decrease is due to membrane energisation and proton pumping leading to the build up of a pH gradient [33]. Addition of ATP, in the dark, to these untreated thylakoid membranes resulted in a rapid decrease in the level of 9-aminoacridine fluorescence (Fig. 2A): this quenching is due to a physical interaction between the ATP and 9-aminoacridine and is not affected by uncouplers; thus it does not reflect the energetic state of the membrane [33]. However, in light-treated trypsin samples (Fig. 2B) addition of ATP resulted in a slow decrease in fluorescence intensity: this decrease was sensitive to tentoxin (see Table I) and the ionophoretic antibiotic gramicidin (data not shown). Similar data were obtained for chymotrypsin and a summary of the results is given in Table I. These results show that both trypsin and chymotrypsin altered the state of the thylakoids in such a way that addition of ATP induced membrane energisation. The inhibition of this effect by tentoxin further suggests that trypsin and chymotrypsin activated ATP hydrolysis and that the reverse proton pumping activity of the ATPase was responsible for the build-up of the pH gradient.

Measurements of photophosphorylation and oxygen evolution were made using thylakoids which had been treated, in the dark or light, with trypsin or chymotrypsin. Table II shows that trypsin had a more marked effect on the light- than on the dark-treated samples: in the dark-treated thylakoids 66% of the control rate of phosphorylation remained after 5 min, whilst in similar light-treated samples complete inhibition occurred (Table II). In addition the results show that the rate of oxygen evolution measured under phos-

TABLE I

THE EFFECTS OF TRYPSIN AND CHYMOTRYPSIN ON ATP HYDROLYSIS AND 9-AMINOACRIDINE FLUORESCENCE

Thylakoids with a chlorophyll concentration of 25 $\mu g \cdot ml^{-1}$ were treated with trypsin (5 $\mu g \cdot ml^{-1}$) or chymotrypsin (15 $\mu g \cdot ml^{-1}$) in the dark or light in a medium containing NaCl (50 mM), K_2HPO_4 (2 mM) and $MgCl_2$ (2 mM) (pH 7.4). On completion of incubation, the light was switched off, trypsin inhibitor (0.5 $mg \cdot ml^{-1}$), methyl viologen (1.0 mM) and where necessary 9-aminoacridine (4 μm) were added. ATP hydrolysis rates and quenching of 9-aminoacridine fluorescence were made by first pre-illuminating the previously treated samples for 1 min, this was followed by 1 min dark after which ATP (0.5 mM) was added.

Incubation time	Hydrolysis rate (nmol ATP per min per mg Chl)		Quenching of 9-aminoacridine fluorescence (%F decreased)	
	Dark	Light	Dark	Light
Trypsin				
Control	0	185 ±22.5 [a]	8.4±0.61	9.6±0.46 [b]
1 min	120.3±27.1 [a]	645 ±56.3 [a]	11.9±0.43 [b]	20.4±1.3 [b]
1 min + tentoxin	–	0	–	9.8±0.41 [b]
Chymotrypsin				
Control	0	152.6±23.0 [a]	8.4±0.61	9.6±0.46
1 min	163.7±36.4 [a]	374 ±33.9 [a]	9.4±0.45 [b]	18.8±0.9 [b]
1 min + tentoxin	–	0	–	8.7±0.37 [b]

[a] The standard errors of a minimum of four determinations was indicated.
[b] The standard errors of a minimum of three determinations was indicated.

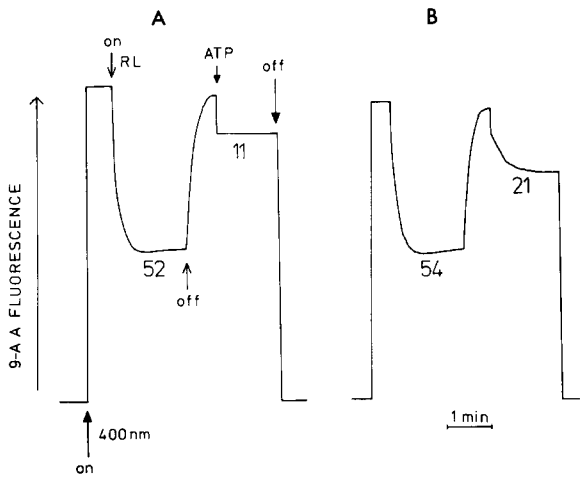

Fig. 2. Effect of ATP on 9-aminoacridine (9-A A) fluorescene in trypsin-treated thylakoid membranes. Thylakoids with a chlorophyll concentration 25 $\mu g \cdot ml^{-1}$ were incubated with trypsin (5 $\mu g \cdot ml^{-1}$) in the dark or light for 1 min in a medium containing NaCl (10 mM), K_2HPO_4 (2 mM) and $MgCl_2$ (2 mM) (pH 7.4). On completion of incubation trypsin inhibitor (0.5 $mg \cdot ml^{-1}$), 9-aminoacridine (4 μM) and methyl viologen (1.0 mM) were added. The actinic light (RL) was switched on for 1 min; this was followed by 1 min dark after which ATP (0.5 mM) was added. (A) Control, no pretreatment; (B) trypsin, 1 min light.

phorylating conditions was inhibited by trypsin treatment, in both dark- and light-treated samples, and after 5 min 82% and 92% of the control rate, respectively, remained. Although the overall effect of trypsin on phosphorylation and oxygen evolution is inhibitory, the data presented in Table II show that after 1 min trypsin treatment in the light both of these parameters are stimulated appreciably. Comparing these data with those presented in Table I it can be seen that the stimulation of phosphorylation occurred under the same conditions which caused a stimulation in ATP hydrolysis. This is an important point in view of the recent findings of Mills and Mitchell [19] who showed that thylakoid membranes with thiol modulated ATPase displayed an increased rate of ATP synthesis and oxygen evolution.

Chymotrypsin inhibited phosphorylation and oxygen evolution (Table II). After 5 min incubation chymotrypsin inhibited phosphorylation, with 58% and 36% of the control rate remaining in the dark- and light-treated samples respectively. The rate of oxygen evolution, measured under phosphorylating conditions, was also inhibited by chymotrypsin: after 5 min incubation in dark-

TABLE II

THE EFFECTS OF TRYPSIN AND CHYMOTRYPSIN ON PHOSPHORYLATION AND OXYGEN EVOLUTION

Phosphorylation and oxygen evolution rates were measured after thylakoids, (chlorophyll concentration, 25 $\mu g \cdot ml^{-1}$) had been treated with trypsin (5 $\mu g \cdot ml^{-1}$) and chymotrypsin (15 $\mu g \cdot ml^{-1}$) in a medium containing NaCl (50 mM), K_2HPO_4 (2 mM) and $MgCl_2$ (2 mM); methyl viologen (1 μM) was added on completion of treatment. All samples were pre-illuminated for 1 min prior to measurements of phosphorylation/O_2 evolution in a manner similar to that described in Table I. ADP (0.5 mM) was then added. The standard errors of a minimum of six determinations are indicated.

Incubation time	Rate of phosphorylation (μmol ATP per mg Chl per h)		Rate of O_2 evolution (μmol O_2 per mg Chl per h)		P/O_2 ratio	
	dark	light	dark	light	dark	light
Trypsin						
Control	170.2±11.3		116 ± 6.4		1.46	
1 min	156.7±113.1	42.2±22.3	116 ±11.2	157 ±16	1.35	1.54
3 min	155.2± 5.9	127.8± 7.6	110 ±18	105 ± 6.4	1.41	1.22
5 min	116.6± 3.6	0	109 ± 6.4	96 ± 6.4	1.07	0
Chymotrypsin						
Control	191.1±28.4		96.9±13.0		1.97	
1 min	169.9±22.5	151.1±26.8	92.4±11.3	89.4±11.1	1.84	1.69
3 min	122.6± 7.4	105.9±16.6	85.4± 8.8	73.4± 4.5	1.43	1.44
5 min	111.2±18.3	68.8± 7.1	71.0± 8.0	64.0± 4.0	1.56	1.07

treated samples 73% remained, whilst in similar light-treated samples 66% remained. These results differ from those of trypsin in two respects: firstly, both phosphorylation and oxygen evolution are inhibited appreciably, and secondly, no stimulation of either of these parameters was evident under any conditions studied.

Discussion

Stimulation of ATP hydrolysis by trypsin and chymotrypsin

Trypsin-treatment of thylakoid membranes has been shown to stimulate ATP hydrolysis [14]; however, it was reported by the same authors that chymotrypsin treatment resulted in the inhibition of hydrolysis. The data presented here (Fig. 1; Table I) clearly demonstrate that, under specific conditions, chymotrypsin can also enhance ATP hydrolysis. The effect of chymotrypsin was shown to have similar characteristics to that shown for trypsin [3] in that light was required during treatment and the activity, which decays in the dark, could be stabilised by the addition of phosphate. In addition tentoxin, an inhibitor of ATP hydrolysis at low concentrations (less than 2 μm) [37], resulted in a loss both of trypsin- and chymotrypsin-stimulated hydrolysis.

Correlation between the decay rate of ΔA_{515} and trypsin- and chymotrypsin-stimulated ATP hydrolysis

Stimulation of ATP hydrolysis by DTT, trypsin and chymotrypsin has been correlated with a marked increase in the rate of decay of ΔA_{515}. This acceleration may be accounted for by the following model: ATP hydrolysis in the dark induces reverse proton pumping [38] which results in the build-up of the transmembrane electrochemical potential gradient. When a flash-induced field is imposed on top of this steady-state electrochemical potential gradient, the driving force for ion movement through the membrane is larger than in the control, hence the electrochemical gradient decays more rapidly [39,40]. The results obtained from the 9-aminoacridine fluorescence experiments (Table I) support this model by showing that, on completion of trypsin- or chymotrypsin-treatment in the light for 1 and 3 min, respectively, addition of ATP causes a build up of a pH gradient across the membrane brought about by the reverse proton pumping linked to ATP hydrolysis.

Stimulation of ATP synthesis by trypsin

Thiol activation of the ATPase complex, in addition to activating ATP hydrolysis, has been shown to increase the rate of steady-state phosphorylation [19]. It was proposed by Mills and Mitchell [19] that this change in the activity of the ATPase was brought about by a lowering of the pH threshold over which the complex is active. Further support for this proposal has come from Morita et al. [39] and Junesch and Graber [20]. The data presented here have shown that trypsin altered the functions of the ATPase complex in a manner similar to thiol activation: both the rates of steady-state phosphorylation and ATP hydrolysis were stimulated. Moroney and McCarty [15] suggested that trypsin-induced stimulation of ATP hydrolysis was due both to activation of the ATPase and uncoupling of the thylakoid membranes. In contrast our data indicate (Table II) that trypsin increased the rate of oxygen evolution under conditions which stimulated both ATP synthesis and ATP hydrolysis and which resulted essentially in no change in the P/O_2 ratio (this ratio was 1.46 in treated and 1.51 untreated samples). Mills and Mitchell [19] have shown a similar effect of DTT activation on ATP synthesis. But when the same experiment was carried out on membranes which had been uncoupled slightly using NH_4Cl, ATP synthesis was stimulated, and the rate of oxygen evolution remained constant resulting in an increase in the P/O_2 ratio [19]. Taken together, these data indicate that mild trypsin digestion alters the catalytic state of the ATPase complex, and suggest – in contrast to the conclusion of Moroney and McCarty [15] – that the activation of ATP hydrolysis by trypsin is not a by-product of uncoupling, but actually precedes the uncoupling effect.

Comparison of the effects of trypsin and chymotrypsin on ATPase function

Trypsin was shown to increase the rate of ATP synthesis under conditions which caused a stimulation in ATP hydrolysis (Table I). These results are in keeping with the model discussed by Mills [41] which summarises the results obtained with DTT and thiol-modulated ATPases: $\Delta\mu_{H^+}$ activation and thiol-modulated reduction of the enzyme is required to observe ATP synthesis and hydrolysis. In addition, in the thiol-reduced enzyme the rate of ATP synthesis is stimulated because the level of $\Delta\mu_{H^+}$ required for activation is lowered [19,20]. However, despite the fact that chymotrypsin also stimulated ATP hydrolysis no increase in the rate of ATP synthesis was detected (Table I). At first sight this result is not in accord with the Mills model, but the difference may be accounted for by comparing the characteristics of inhibition of the ATPase complex by trypsin and chymotrypsin.

Trypsin-treatment (5 min light) was shown to inhibit ATP synthesis by uncoupling of electron transport from photophosphorylation, in agreement with previous reports [15,42,43]. Chymotrypsin also inhibited phosphorylation in both dark- and light-treated samples, but in contrast to trypsin, oxygen evolution was significantly inhibited by chymotrypsin. These data suggest that the chymotrypsin inhibition of ATP synthesis is more in keeping with the characteristics of an energy-transfer inhibitor than with an uncoupler. Thus it appears that, although chymotrypsin stimulates the hydrolysis reaction of the ATPase complex in a manner superficially similar to that of trypsin, a concomitant stimulation of ATP synthesis is not observed because the direct inhibition of the phosphorylation reaction by chymotrypsin counteracts any stimulation which may be induced.

The use of trypsin has yielded much information on the functions of this complex, but more knowledge has to be gathered before the mechanisms involved in the functions of the ATPase enzyme are fully understood. The results given here suggest that chymotrypsin may be a useful tool to investigate this complex further and to help correlate changes in function with changes in structure of a specific subunit.

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