Evidence for a novel ATP-dependent membrane-associated protease in spinach leaf mitochondria

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We report the presence of an ATP-dependent proteolytic activity in spinach (*Spinacia oleracea*) leaf mitochondria. The proteolysis was observed as degradation of newly imported precursor protein. The precursor studied was that of the ATP synthase $F_1\beta$ subunit of *Nicotiana plumbaginifolia*, transcribed and translated *in vitro*. Degradation of pre- $F_1\beta$ was observed during kinetic studies of import *in vitro*. The degradation was characterized in chase experiments in which the precursor was imported into mitochondria. The import reaction was subsequently stopped by the addition of valinomycin and oligomycin. The fate of the imported precursor inside the mitochondria was monitored under different experimental conditions. There was no proteolytic degradation of the newly imported precursor at 15 °C, whereas

50 % of the precursor was degraded after a 45 min incubation at 25 °C. The proteolytic activity was found to be ATP-dependent and was partially inhibited by a metal chelator, *o*-phenanthroline. Fractionation of mitochondria prior to degradation showed that all the ATP-dependent degradative activity was associated with the mitochondrial membrane fraction. The membrane-bound protease was inhibited by Pefabloc [4-(2-aminoethyl)-benzenesulphonyl fluoride hypochloride], an inhibitor of serine-type proteases and by *N*-ethylmaleimide, a thiol group reagent. Our studies thus describe a novel ATP-dependent membrane-associated serine-type protease in plant mitochondria that is capable of degrading newly imported non-assembled proteins.

INTRODUCTION

The existence of mitochondria and the sustenance of mitochondrial function depends upon co-ordinated protein synthesis and protein degradation. The mitochondrion contains its own genome and its own transcription/translation machinery. The mitochondrial DNA encodes a number of membrane-bound proteins of the respiratory chain complexes and of the ATP synthase complex. However, the vast majority of the mitochondrial proteins are encoded in the nuclear genome. Nuclear-encoded mitochondrial proteins are translated in the cytosol as precursor proteins and subsequently imported into the organelle. After import into the organelle, the precursor is processed to a mature size product by a processing peptidase [1,2]. The concerted action of the mitochondrial chaperones mthsp70 [3], DnaJ [4], GrpE [5] and mthsp60 [6], together with hsp10 [7], mediates the assembly of the new protein into functional protein complexes. All of the oligomeric protein complexes of the mitochondrial inner membrane consist partially or entirely of nuclear-encoded proteins.

The regulation of the biosynthesis of these complexes can take place in different ways, either at the level of strict transcriptional and translational co-ordination of the expression of the two genomes or by the degradation of proteins which are in excess and thus not assembled. It has been shown that the expression levels of nuclear-encoded mammalian mitochondrial proteins are not strictly co-ordinated with each other [8]. Studies of the assembly of the mitochondrial bc_1 complex, of the F_0F_1 -ATPase in yeast [9,10] and of the chloroplast *bf* complex in *Lemna* [11] show that when one subunit of the complex is absent the other subunits, although translated, are not stable and only small amounts of proteins can be found in the organelle. The stability

of the proteins is dependent on their location within the complex in relation to the absent protein. Another study with yeast, using a genetic depletion strategy [12], has demonstrated that in order to assemble the imported precursor of ATP synthase subunit 8 into a functional complex, pre-existing subcomplexes ready to incorporate the protein are required. There are a variety of other reasons why mitochondrial proteases would degrade a specific part of the mitochondrial protein complement: enzymic or oxidative damage, regulation of activity by changing the amount of enzyme or fine tuning of biogenesis if some subunits are present in excess amounts.

The degradation of damaged, misfolded or non-assembled proteins in mitochondria has been briefly characterized [13]. It has been shown that, during *in organello* protein synthesis in yeast mitochondria, extensive ATP-dependent degradation of normal but non-assembled translation products occurs [14]. Mitochondria have been shown to contain two types of ATP-dependent proteases. The yeast *PIM1* gene encodes a protein that shows 30 % sequence identity to the La(*lon*) protease of bacteria [15,16]. This protein is an ATP-dependent serine-type protease located in the matrix and is inducible by heat shock [17]. This protein acts together with the mitochondrial chaperones hsp70 and Mdj1p (an analogue of the bacterial DnaJ) [18]. Another protease, Yta 10p, has recently been characterized in yeast [19,20]; it is also ATP-dependent and related to the bacterial FtsH protease [19]. The Yta 10p protein is an integral inner membrane protein; it might be the same protease as that recently characterized biochemically from yeast mitochondria by Yasuhara et al. [21].

In the present study we have investigated the fate of the newly imported precursor of *Nicotiana plumbaginifolia* $F_1\beta$ subunit of the ATP synthase (pre- $F_1\beta$) in spinach leaf mitochondria. We

Abbreviations used: NEM, *N*-ethylmaleimide; Pefabloc, 4-(2-aminoethyl)benzenesulphonyl fluoride hypochloride; PK, proteinase K; mthsp70, Mdj 1p, GrpE, hsp10 and mthsp60, mitochondrial chaperones; MPP, mitochondrial processing peptidase.

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found that the imported precursor is subject to ATP-dependent proteolysis. We have characterized the proteolytic activity with respect to its intramitochondrial location and properties.

MATERIALS AND METHODS

Growth conditions and isolation of *Spinacia oleracea* mitochondria

Spinach (*Spinacia oleracea* L. cv. Medania) was hydroponically grown as described by Siegenthaler and Depsy [22] under artificial light at 25 C. Spinach leaf mitochondria were prepared from 6-week-old leaves with the mid-vein removed. Mitochondria were isolated by density step centrifugation according to Knorpp et al. [23]. Protein content was measured according to Bradford [24].

In vitro synthesis of the *N. plumbaginifolia* F₁ precursor

A cDNA clone of the F₁ subunit of the ATP synthase from *N. plumbaginifolia* was maintained in a pTZ 18U vector. *In vitro* transcription from the vector's T7 promoter and translation were performed in a coupled reticulocyte lysate system (SDS-Promega) in the presence of [³⁵S]methionine (Amersham) according to the manufacturer's recommendations.

Import of the F₁ precursor into mitochondria

Import experiments were carried out using 200 g of mitochondrial protein in a final volume of 200 l of medium containing 0.23 M mannitol, 50 mM KCl, 10 mM Mops, 1 mM ATP, 10 M ADP, 1 mM methionine, 1 mM glycine, 5 mM K₂PO₄ and 1 % (w/v) BSA, pH 7.4 (import buffer). The amount of precursor protein used in import experiments corresponded to 40000 c.p.m. Import was performed by incubation of radio-labelled pre-F₁ with mitochondria at different temperatures and times. The reaction was stopped on ice. In order to remove the precursor which had not been imported, samples were incubated with proteinase K (PK) at 4 g/ml for 30 min on ice. The proteolytic reaction was stopped by the addition of PMSF to 2 mM. After PK treatment the mitochondria were re-isolated by a 5 min centrifugation at 10000 rev./min in a Microfuge and lysed in the sample buffer of Laemmli [25]. Samples were analysed by SDS/PAGE using 12 % (w/v) polyacrylamide gels in the presence of 4 M urea [25]. The gels were subsequently fixed and impregnated with Amplify (Amersham), dried, fluorographed and scanned using a Molecular Dynamics Personal Densitometer. Import was measured as the amount of the mature form of pre-F₁ present inside mitochondria after PK treatment.

Degradation of the F₁ precursor in mitochondria

Degradation of newly imported pre-F₁ was studied in chase experiments in which the precursor was imported into spinach leaf mitochondria for 15 min at 25 C as described above. The time and temperature were chosen in order to obtain the maximal amount of imported protein present inside the mitochondria ([26] and Figure 1). Import was stopped by the addition of 10 M valinomycin and 20 M oligomycin, and the mitochondria were pelleted as described above. The mitochondria were then resuspended in import buffer. Degradation of the imported pre-F₁ was studied during the further incubation of the mitochondria under different experimental conditions. Degradation was studied in the presence of 1 mM ATP at 15 C or 25 C. The degradation at 25 C was also studied in the presence of 40 units/ml apyrase, which removes ATP, and also with 1 mM ATP and different inhibitors such as 25 mM *o*-phenanthroline and 1 mM *N*-ethylmaleimide (NEM). The re-

actions were stopped on ice and the samples treated with PK as described above. For fractionation, the mitochondria containing newly imported precursor protein were pelleted in a Microfuge, resuspended in 30 l of 0.3 M sucrose, 30 mM MgCl₂, 10 mM Mops, pH 7.5, and 2 mM PMSF, and sonicated in a water-bath sonicator (Ultrasonic) for 3 30 s at 4 C with an interval of 30 s between sonications. Mitochondrial fractions were separated by centrifugation at 105000 g for 45 min. The membrane and matrix fractions were analysed by SDS/PAGE and fluorography as described above.

Degradation of the F₁ precursor by different mitochondrial fractions

Mitochondria were diluted in 0.3 M sucrose, 30 mM MgCl₂ and 10 mM Mops, pH 7.5, to a final protein concentration of 10 mgml⁻¹ and sonicated using a Branson Sonifier (equipped with a microtip; setting 3) for 3 30 s at 4 C with an interval of 30 s between sonications. The sonicated mitochondria were centrifuged at 105000 g for 45 min and both the membrane and the matrix fractions were used in degradation experiments. The volume of the matrix was measured and the membranes were resuspended in an equal volume of 0.3 M sucrose, 10 mM Mops, pH 7.5. Assays of degradation contained 12 l of matrix or membranes and an equal volume of 30 mM Tris/HCl pH 8.0, 1 % (w/v) Triton X-100, 1.0 mM MnCl₂, 1.0 mM methionine and 0.5 l (10000 c.p.m.) of [³⁵S]methionine-labelled *N. plumbaginifolia* pre-F₁. The degradation reaction was followed in the presence (1 mM) or absence (40 units/ml apyrase) of ATP. The effects of inhibitors [2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride hypochloride (Pefabloc), 25 mM *o*-phenanthroline, 1 mM NEM, 0.1 mM haemin or 5 mM vanadate] were investigated in the presence of 1 mM ATP. After incubation the reaction was stopped with double-strength sample buffer of Laemmli [25] and placed on ice. Samples were analysed by SDS/PAGE and fluorographed as described above.

Dissociation of the ATP-dependent protease from the membranes

The isolated mitochondrial membranes were washed in 0.1 % Triton X-100, 0.3 M sucrose, 10 mM Mops, pH 7.5, at a protein concentration of 4 mgml⁻¹ and immediately re-isolated. The resulting supernatant and pellet fractions were tested for degradative activity as described above.

Chemicals

The following reagents were purchased from Sigma: ATP, Pk, PMSF, oligomycin, valinomycin, haemin, *o*-phenanthroline, NEM, apyrase and vanadate. Pefabloc was purchased from Boehringer Mannheim.

RESULTS

Figure 1 shows the kinetics of the *in vitro* import of pre-F₁ into spinach leaf mitochondria at 15 C and 25 C. The choice of temperature was based on a previous study where we investigated the temperature optima of import of pre-F₁ into spinach leaf mitochondria [23]. We found two temperature optima, at 15 C and 25 C. The kinetic measurements of import at 15 C showed a saturation-type curve, which reached a maximum after 15 min. At 25 C the import was faster and the amount of imported protein was higher in the initial phase of the experiment than at 15 C. The maximum level of imported protein was reached at 15 min, whereafter it started to decrease. After 45 min of *in vitro* import, 50 % of the imported protein that was present inside the mitochondria at 15 min had been degraded. When ATP, normally

Figure 1 Kinetics of import of *N. plumbaginifolia* $F_1\beta$ into spinach leaf mitochondria at different temperatures

Import was performed at 15 °C (○) or at 25 °C (●) in the presence of 1 mM ATP, or at 25 °C in the presence of apyrase (40 units/ml) (▲). Values are means of two to four experiments; S.D.s were less than 10%.

Figure 2 Import and degradation of *N. plumbaginifolia* $F_1\beta$ in spinach leaf mitochondria at 15 °C and 25 °C

Import of the precursor protein into mitochondria was performed for 15 min at 25 °C and then stopped by the addition of oligomycin and valinomycin (see the Materials and methods section). Degradation was followed during a further incubation at 15 °C (○) or 25 °C (●). The amount of imported protein after 15 min of import corresponds to 100%. Values are means of four to six experiments; S.D.s were less than 10%.

present at a concentration of 1 mM, was removed by the addition of apyrase (which hydrolyses the available pool of ATP) both import and degradation were affected. The kinetics of import at 25 °C in the absence of ATP were similar to those at 15 °C in the presence of ATP (Figure 1).

In order to separate the import process from the degradation, we used chase experiments (see the Materials and methods section) and investigated the effects of temperature on import and degradation (Figure 2) and the effects of ATP and protease inhibitors on degradation (Table 1). Degradation was measured as the amount of PK-protected mature form of the precursor that

Table 1 Degradation of *N. plumbaginifolia* $F_1\beta$ in spinach mitochondria in the presence of protease inhibitors

The effects of protease inhibitors on the degradation of imported pre- $F_1\beta$ in spinach leaf mitochondria were studied at 25 °C in chase experiments as described in the Materials and methods section. The amount of protein degraded in the presence of 1 mM ATP corresponds to 100%. Chase experiment were performed in the presence of 40 units/ml apyrase, 25 mM *o*-phenanthroline or 1 mM NEM. Values represent the amount of pre- $F_1\beta$ degraded inside the mitochondria after 45 min of incubation with the inhibitors, and are means $\pm$ S.D. of four experiments.

Inhibitor	Activity (%)
Control	100 $\pm$ 9
Apyrase	12 $\pm$ 1
<i>o</i> -Phenanthroline	47 $\pm$ 3
NEM	86 $\pm$ 2

Figure 3 Intramitochondrial location of imported *N. plumbaginifolia* $F_1\beta$

Import and chase experiments were performed as described in Figure 2. Lane 1, *in vitro* synthesized $F_1\beta$ precursor protein; lanes 2 and 5, imported pre- $F_1\beta$ after 15 and 60 min of incubation respectively; lanes 3 and 6, $F_1\beta$ present in the matrix fraction after 15 and 60 min respectively; lanes 4 and 7, $F_1\beta$ present in the membrane fraction after 15 and 60 min respectively. p, pre- $F_1\beta$; m, mature $F_1\beta$.

disappeared during the course of the chase experiment. Figure 2 shows that the imported protein was degraded inside the mitochondria at 25 °C but not at 15 °C. Table 1 shows the effects of protease inhibitors on degradation measured in chase experiments. The percentage of the imported protein that was degraded after 35 min of chase at 25 °C in the presence of ATP was taken as 100% degradation. Addition of apyrase inhibited almost 90% of this activity. The remaining activity could be due to residual ATP, which is difficult to remove from the intact organelle. *o*-Phenanthroline, a known metal chelator, inhibited 47% of the degradation activity, while NEM, a thiol group reagent, inhibited only 14%. We conclude that the proteolytic enzyme responsible for the degradation of newly imported pre- $F_1\beta$ is strictly ATP-dependent and is directly or indirectly dependent on a metal.

In order to determine the intramitochondrial location of the substrate for ATP-dependent proteolysis and to investigate whether different pools of the substrate are equally degraded, we imported pre- $F_1\beta$ into mitochondria and monitored the location of the imported protein before and after degradation, as described in the Materials and methods section (Figure 3). The mitochondria were sonicated after the completed experiment and the matrix and membrane fractions were separated and analysed. We observed that 40% of the imported protein was found in the membrane fraction and 60% was in the matrix. The relative proportions of the imported protein that were recovered in the membrane and matrix fractions did not change during the chase experiment. No firm conclusions could be drawn concerning the location of the imported substrate. We then decided to investigate the distribution of the proteolytic activity. We separated the matrix from the membranes by sonication and incubated the fractions with pre- $F_1\beta$. Degradation was estimated as the amount

Figure 4 Degradation of *N. plumbaginifolia* $F_1\beta$ by spinach mitochondrial fractions in the presence and absence of ATP

N. plumbaginifolia $F_1\beta$ precursor was incubated with mitochondrial fractions at 30 °C. Incubation was stopped after 5, 30 and 60 min with Laemmli sample buffer [25]. (a) Degradation of pre- $F_1\beta$ by membranes in the presence of 1 mM ATP; (b) degradation of pre- $F_1\beta$ by matrix in the presence of 1 mM ATP; (c) degradation of pre- $F_1\beta$ by membranes in the presence of 40 units/ml apyrase; (d) degradation of pre- $F_1\beta$ by matrix in the presence of 40 units/ml apyrase. p, pre- $F_1\beta$; m, mature $F_1\beta$.

Table 2 ATP-dependent degradation of *N. plumbaginifolia* $F_1\beta$ by spinach mitochondrial membranes in the presence of protease inhibitors

Experimental conditions are described in the Materials and methods section. The concentrations of the inhibitors were as follows: 40 units/ml apyrase, 2 mM Pefabloc, 25 mM *o*-phenanthroline, 1 mM NEM, 0.1 mM haemin and 5 mM vanadate. Degradation in the presence of 1 mM ATP corresponds to 100%. Values are means $\pm$ S.D. average of four to six experiments.

Inhibitor	Activity (%)
Control	100 $\pm$ 4
Apyrase	1 $\pm$ 5
Pefabloc	37 $\pm$ 4
<i>o</i> -Phenanthroline	81 $\pm$ 10
NEM	18 $\pm$ 4
Haemin	100 $\pm$ 1
Vanadate	94 $\pm$ 2

of the precursor and/or mature form that disappeared between 5 and 60 min of incubation. Figure 4 shows results of the incubation of pre- $F_1\beta$ with membranes and matrix fractions in the presence or absence of ATP. Samples were removed after 5, 30 and 60 min and analysed. Several interesting observations can be made. The membrane fraction exhibited extensive degradation in the presence of ATP (Figure 4a). When ATP was removed by the addition of apyrase (Figure 4c), the degradation activity was almost completely abolished. No degradation could be observed in the matrix fraction (Figure 4b). The proteolytic activity that gives the intermediate form found in the matrix differs from the activity that is catalysed by the general mitochondrial processing peptidase (MPP), which is integrated into the bc_1 complex of the respiratory chain [27]. Unlike MPP, the matrix proteolytic activity was sensitive to Pefabloc (C. Szigyarto, C. Knorpp and E. Glaser, unpublished work). Under our experimental conditions, all of the ATP-dependent degradation observed with the fractionated mitochondria was associated with the membrane fraction. The membrane-associated degradation activity was further characterized using different protease inhibitors (Table 2). In agreement with the chase experiments (cf. Table 1), the degradation was shown to be strictly ATP-dependent and was partially inhibited by *o*-phenanthroline. It was also evident that the membrane-associated protease was sensitive to a serine

Table 3 Dissociation of the ATP-dependent protease from mitochondrial membranes

Membranes were isolated and washed in the presence of 0.1% (v/v) Triton X-100 and the degradation of $F_1\beta$ was studied as described in the Materials and methods section, in either the absence or the presence of 2 mM Pefabloc. Degradation by membranes before the washing procedure in the presence of ATP corresponds to 100%. Values are means $\pm$ S.D. of two to six experiments.

Mitochondrial fraction	Pefabloc	Activity (%)
Membranes	—	100 $\pm$ 4
	+	37 $\pm$ 4
Supernatant	—	41 $\pm$ 1
	+	0 $\pm$ 4
Pellet	—	58 $\pm$ 1
	+	18 $\pm$ 2

protease inhibitor, Pefabloc, and to a cysteine reagent, NEM. No effect was seen with the protease inhibitors hemin and vanadate. Hemin is a powerful inhibitor of the membrane protease studied by Yasuhara et al. [21] and vanadate is a characteristic inhibitor of La-like proteases [13].

In order to determine if the ATP-dependent proteolytic activity was associated with an integral or a peripheral membrane protein or protein complex, we performed a relatively mild detergent wash of the membrane fraction. The membranes were suspended in a medium containing 0.1% Triton X-100 and immediately re-isolated. The resulting supernatant and pellet were tested for the degradation activity. The pellet contained 60% of the control activity and 40% of the activity could be observed in the supernatant (Table 3). These results indicate that the ATP-dependent degradation protease is rather loosely bound to the mitochondrial membrane. Both the activity in the pellet and that released into the supernatant could be inhibited by Pefabloc.

DISCUSSION

In this paper we have characterized an ATP-dependent degradative activity in spinach leaf mitochondria that catalyses the degradation of a newly imported precursor protein. As substrate we have used a plant nuclear-encoded mitochondrial subunit, the precursor of *N. plumbaginifolia* ATP synthase $F_1\beta$. We have shown that the newly imported pre- $F_1\beta$ is degraded at increased temperatures, and that the degradation activity is partially sensitive to a metal chelator, *o*-phenanthroline. Studies with isolated mitochondrial fractions showed that the protease is associated with the mitochondrial membrane. The results also suggest that the enzyme is a serine-type protease and that thiol groups are involved at some stage of the proteolytic process.

Many of the ATP-dependent proteases have been shown to be dependent on metal ions. Some proteases are metalloendopeptidases such as, for example, bacterial thermolysin [29] and mitochondrial Yta 10p protease [20], which both carry a consensus binding site for bivalent metal ions [30]. If a metal were to be a part of the catalytic site in our system, the observed partial metal-dependence could be the result of inaccessibility of *o*-phenanthroline to the catalytic site. Another possibility is that a metal is used indirectly by the protease, e.g. for binding of Mg-ATP [15].

Both the bacterial La and Ti proteases [13] and their mitochondrial analogue, the *PIM1* protease [16,18], are serine proteases which contain an essential thiol group [31]. The mitochondrial chaperone Mdj1p, which is involved in the degradation

of misfolded proteins, also has an essential thiol group [18]. Pefabloc, a novel powerful serine protease inhibitor, inhibited the spinach ATP-dependent protease under conditions where the inhibitory effect was investigated directly with the isolated membrane fraction. The inhibitory effect of NEM on the degradation was small in intact mitochondria but much more pronounced when investigated with the isolated membranes (cf. Tables 1 and 2). Like *PIMI* protease, the ATP-dependent plant protease uses newly imported, non-assembled and probably misfolded proteins as substrates. These results indicate that the properties of the spinach ATP-dependent protease resemble, to some extent, the properties of the *PIMI* protease [16,18]. The *PIMI* protease is, however, located in the mitochondrial matrix, while the spinach protease in our system was shown to be membrane-associated. We note that the plant chloroplast clpA protein, for which no mitochondrial equivalent has been found, has been observed in a membrane fraction [32]. The bacterial La and Ti proteases have been shown to be sensitive to vanadate [13] which is not the case for the plant protease investigated in our system. We thus conclude that the plant protease is not identical to *PIMI*.

Many events in the mitochondrial protein import process, especially in plants, are membrane-associated. The general processing peptidase in plants is an integral part of the *bc₁* complex of the respiratory chain [27,33–35]. The rest of the import apparatus is also believed to be membrane-associated, up to the stage where mthsp60 (in concerted action with hsp10 [35]) assists assembly [36,37]. A proportion (60%) of the spinach leaf mitochondrial hsp70 has been shown to be associated with the inner membrane [38]. The binding of hsp70 to the mitochondrial inner membrane import apparatus [38] is believed to be a dynamic process involving cyclical binding of mthsp70 to imported precursors followed by ATP hydrolysis and dissociation of the chaperone from the precursor. An appealing hypothesis is that the newly imported pre-*F₁* β is associated with mthsp70 and is presented to the protease in this state. This would also explain the partial localization of the newly imported precursor in the membrane and indicate that the ATP-dependent proteolysis is probably taking place at the boundary between the inner membrane and the matrix.

In our experiments we have imported one of the four nuclear-encoded subunits of the *F₁*-oligomer in the absence of the other subunits. This may lead to the formation of a subcomplex between the mitochondrially encoded *F₁* α subunit and the newly imported *F₁* β subunit, and/or to the presence of a completely non-assembled and probably misfolded *F₁* β precursor inside the mitochondria. Insertion of the newly imported *F₁* β subunit into an already existing *F₁* complex would require specific degradation of the already assembled *F₁* β subunits and seems less plausible, especially considering the results of Law et al. [12], who demonstrated that in order to assemble the imported precursor of ATP synthase subunit 8 into functional complex, pre-existing subcomplexes ready to incorporate the protein were required.

On the basis of the above results, we suggest that the protease characterized in our system is responsible for degradation of non-assembled imported proteins. The *PIMI* protease (the La protease analogue) located in the mitochondrial matrix has been shown to fulfil this function in yeast [16,18]. However, as discussed above, the plant protease differs from the *PIMI* protease in several respects, and no specific immunological cross-reactivity could be observed in spinach mitochondria with antibodies raised against mammalian or yeast La-type proteases (results not shown). Mitochondrially synthesized proteins have

been shown to be degraded by membrane-bound proteases in yeast [19–21]. The plant protease differs from these yeast membrane proteases [20,21] in that it is loosely bound to the membrane and is inhibited by inhibitors of serine-type proteases and not by hemin [19]. Our results point to the conclusion that the observed ATP-dependent degradation in plant mitochondria is catalysed by a novel membrane-associated protease that degrades non-assembled proteins.

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