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Inner membrane protease I, an enzyme mediating intramitochondrial protein sorting in yeast

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Several precursors transported from the cytoplasm to the intermembrane space of yeast mitochondria are first cleaved by the *MAS*-encoded protease in the matrix space and then by additional proteases that have not been characterized. We have now developed a specific assay for one of these other proteases. The enzyme is an integral protein of the inner membrane; it requires divalent cations and acidic phospholipid for activity, and is defective in yeast mutant *pet ts2858* which accumulates an incompletely processed cytochrome b_2 precursor. The protease contains a 21.4 kd subunit whose C-terminal part is exposed on the outer face of the inner membrane. An antibody against this polypeptide inhibits the activity of the protease. As overproduction of the polypeptide does not increase the activity of the protease in mitochondria, the enzyme may be a hetero-oligomer. This 'inner membrane protease I' shares several key features with the leader peptidase of *Escherichia coli* and the signal peptidase of the endoplasmic reticulum.

Key words: cytochrome b_2/cytochrome oxidase subunit II/ intermembrane space/*PET2858*

Introduction

Transport of proteins across prokaryotic and eukaryotic membranes is usually accompanied by proteolytic removal of the targeting sequences from the transported precursor polypeptides (Verner and Schatz, 1988). While some of the corresponding proteases have been well characterized (Zwizinski *et al.*, 1981; Bilgin *et al.*, 1990; Evans *et al.*, 1986; Baker and Lively, 1987; Hawlitschek *et al.*, 1988; Yang *et al.*, 1988), others remain poorly defined. For example, transport of pre-cytochrome b_2 (pre-cytb_2) from the cytoplasm to the soluble intermembrane space of yeast mitochondria involves two proteolytic steps. In the first step, the *MAS*-encoded protease removes the N-terminal matrix-targeting signal in the soluble matrix space, generating a transmembrane cytb_2 intermediate; in the second step, an unknown protease removes the hydrophobic sorting sequence from the intermediate, thereby releasing the mature cytb_2 into the soluble intermembrane space (Daum *et al.*, 1982; Gasser *et al.*, 1982; Hurt and van Loon, 1986).

Pratje and coworkers have identified a yeast mutant in which the activity of this protease appears to be temperature-sensitive (Pratje *et al.*, 1983; Pratje and Guiard, 1986). This mutant (termed *pet ts2858*) accumulates not only the incompletely processed cytb_2 intermediate, but also the precursor form of cytochrome oxidase subunit II. Cytochrome oxidase subunit II is synthesized as a precursor inside the mitochondria and undergoes a single cleavage during its insertion into the inner membrane (Sevarino and Poyton, 1980; Pratje *et al.*, 1983). This suggests that a single enzyme (which we now term inner membrane protease I) mediates proteolytic processing of polypeptides imported from the cytoplasm, or made inside the mitochondria. Mutant *pet ts2858* was a promising experimental basis for identifying and isolating this protease. The wild-type allele of the nuclear gene defective in the *pet ts2858* mutant (termed *PET2858*) potentially encodes a 21.4 kd protein with partial sequence identity to *Escherichia coli* leader peptidase (M.Behrens, G.Michaelis and E.Pratje, submitted).

Here we report a specific *in vitro* assay for the protease and the solubilization of the active enzyme from yeast mitochondria. We identify the submitochondrial localization of the protease, describe its metal and phospholipid requirements, and show that the *PET2858* gene encodes a subunit of the enzyme.

Results

An in vitro assay for inner membrane protease I

In order to study the protease, we had to work out an assay for the solubilized enzyme. Extracts of yeast mitochondria prepared with a variety of non-ionic detergents failed to generate mature cytb_2 from *in vitro*-synthesized pre-cytb_2. The extracts were also inactive towards the cytb_2 intermediate which had been generated from pre-cytb_2 by incubation with purified matrix protease (not shown). This suggested to us that the conformation of these *in vitro*-synthesized substrates differed from that of the cytb_2 intermediate in intact mitochondria. Accordingly, we used as a substrate a detergent extract of mitochondria from mutant *pet ts2858* which accumulates the cytb_2 intermediate and thus appears to be deficient in the inner membrane protease I. When an extract of these mutant mitochondria was incubated with an extract of wild-type mitochondria, the cytb_2 intermediate derived from the mutant mitochondria was converted to mature cytb_2. However, as this assay was based on immunoblotting, it was complicated by the presence of mature cytb_2 in the wild-type mitochondria which were used as a source of enzyme. In order to eliminate this background, the gene coding for cytb_2 in the wild-type strain was disrupted. Mitochondrial extracts from this cytb_2-less strain allowed us to monitor cleavage of the cytb_2 intermediate and were thus routinely used as a source of the inner membrane protease I (Figure 1).

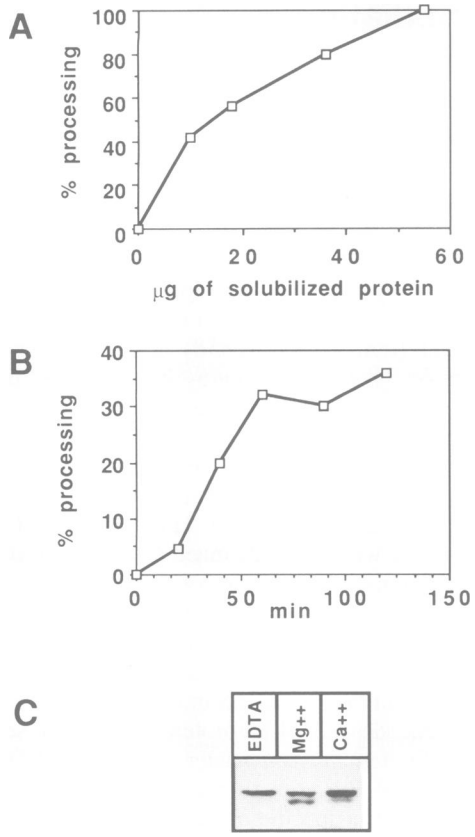

Fig. 1. The cleavage assay. **A.** Cleavage is approximately linear with the amount of enzyme. Standard assay conditions. **B.** Cleavage is linear with time. 300 µg of cytb_2-free mitochondria were incubated at 30°C in standard buffer and 40 µg aliquots were analyzed at the indicated time points. Incubation of the substrate mitochondria without enzyme does not give any signal. **C.** Cleavage is Mg^{2+}-dependent. A constant amount of cytb_2-free mitochondria (40 µg) was assayed for 1 h at 30°C either in standard buffer (containing 5 mM $MgCl_2$) or in standard buffer with the following modifications: (a) no $MgCl_2$, 5 mM EDTA; (b) no $MgCl_2$, 5 mM $CaCl_2$.

Optimization of the assay

Among the detergents tested [Triton X100, CHAPS, deoxycholate, octylglucoside and octyl-polyoxyethylene (octyl-POE)], octylglucoside and octyl-POE yielded the most active mitochondrial extracts (not shown). Octyl-POE was chosen for all further work. Concentrations of 0.4% in the assay were optimal; higher concentrations inhibited the enzyme. This inhibition was reversible as shown by the fact that the enzyme could be solubilized by 1% octyl-POE without loss of activity, as long as the detergent was diluted to ≤0.4% in the assay reaction. Octyl-POE concentrations above 1%, however, caused irreversible loss of activity.

Linearity of the assay with respect to amount of enzyme and time is shown in Figure 1A and B. The protease was inhibited by EDTA and stimulated by Mg^{2+}, Ca^{2+} (Figure 1C) or Mn^{2+}. Zn^{2+} and N-ethylmaleimide (NEM) caused irreversible inactivation (not shown). The optimal Mg^{2+} concentration was ~10 mM. At 30°C, the temperature chosen for the standard assay, the inner membrane protease I of mutant *pet ts2858* was only partly inactivated (not shown). In order to eliminate any background activity, the mitochondrial extracts from mutant *pet ts2858* were routinely pretreated with 10 mM NEM (see Materials and methods).

We did not observe a distinct pH optimum; activity did not vary much between pH 5.5 and 8.5 (not shown).

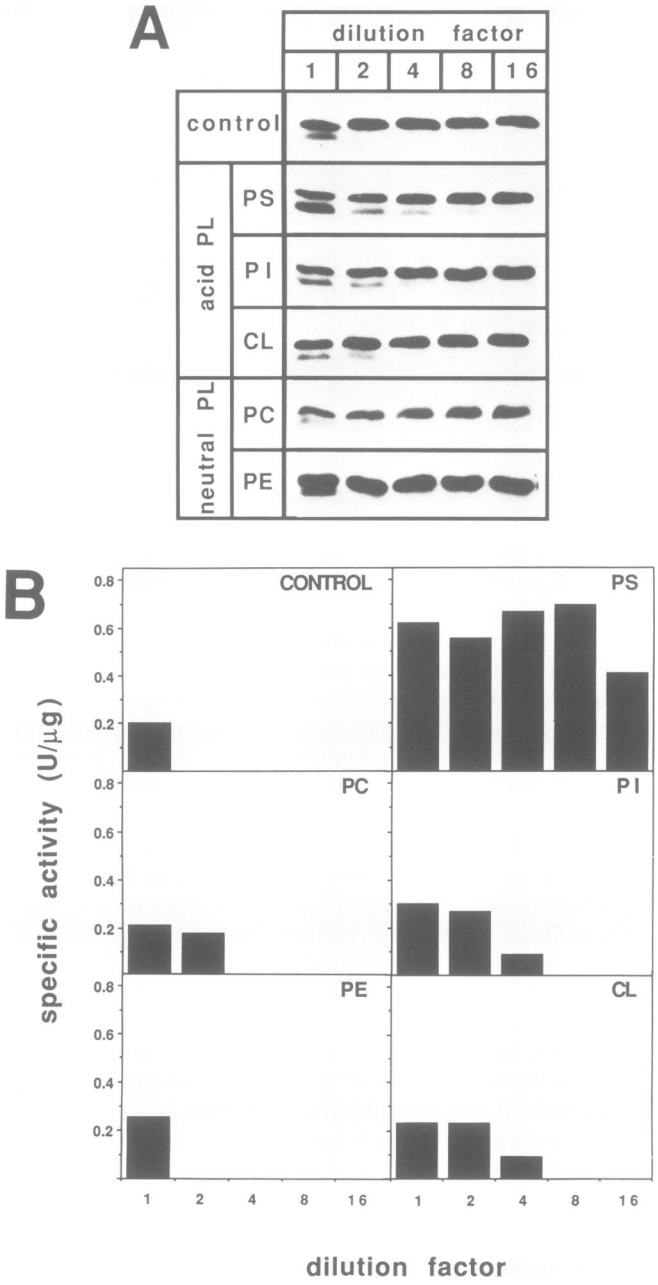

Fig. 2. The cleavage activity requires acidic phospholipid. **A.** Serial dilutions (90, 45, 22, 11 and 5.5 µg) of solubilized mitochondrial membranes (see Materials and methods) were assayed for cleavage activity for 1 h at 30°C in standard buffer either lacking phosphatidyl serine or containing 0.5 mg/ml of the indicated phospholipids. This concentration of phospholipid was the highest concentration which did not inhibit the reaction. **B.** The gels shown in A were scanned to quantify the conversion of cytb_2 intermediate to mature cytb_2 and the specific activities were calculated for each dilution. The detection limit for scanning was 5% cleavage of cytb_2 intermediate. PL, phospholipids; PS, phosphatidyl serine; PI, phosphatidyl inositol; CL, cardiolipin; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

One unit of activity is defined as the amount of enzyme which cleaves 50% of the cytb_2 intermediate to mature cytb_2 under the standard assay conditions.

The protease requires acidic phospholipid

Dilution of the detergent extract caused loss of enzyme activity unless acidic phospholipids were added (Figure 2). Phosphatidyl serine was the most active, followed by

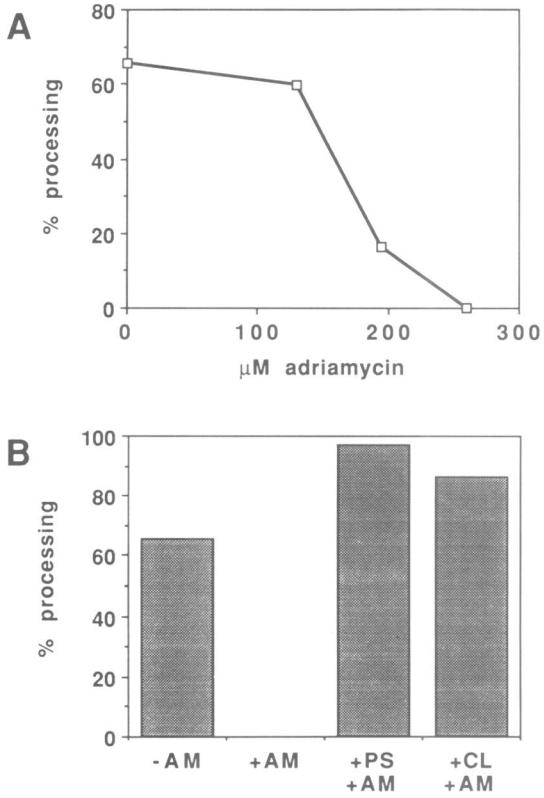

Fig. 3. Adriamycin inhibits the cleavage activity. **A.** The indicated amounts of adriamycin were added to the assay reaction containing 150 μg of solubilized mitochondrial membranes. The reactions were carried out under standard conditions except that no phospholipids were added. **B.** Effect of 260 μM adriamycin on cleavage activity in the absence and presence of added phospholipids. The amount of solubilized mitochondrial membranes was 150 μg. −AM, no adriamycin. +AM, plus adriamycin. +PS, +AM, phosphatidyl serine was added to 0.5 mg/ml, followed by adriamycin. +CL, +AM, cardiolipin was added to 0.5 mg/ml, followed by adriamycin.

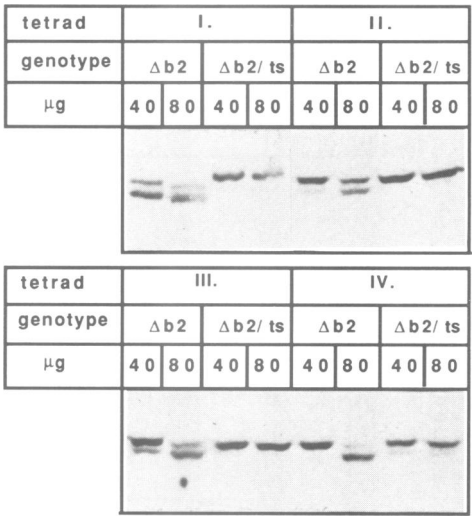

Fig. 4. Mutant *pet ts2858* lacks cleavage activity. Mutant *pet ts2858* was crossed with a strain carrying the wild-type allele of *ts2858*, but lacking a functional gene for cytb_2 (Δb_2); the resulting diploids were induced to sporulate. From each of four tetrads (I–IV), two cytb_2-deficient spores were selected: one of them also carried the *pet ts2858* allele (Δb_2/ts), the other one did not (Δb_2). Aliquots (40 and 80 μg) of mitochondria from these two offsprings were tested for cleavage activity with the cytb_2 intermediate as the substrate under the standard assay conditions.

phosphatidyl inositol and cardiolipin; the neutral phospholipids phosphatidyl choline and phosphatidyl ethanolamine were essentially inactive. Phosphatidyl serine also stimulated the activity of the undiluted extract. The concentration of phosphatidyl serine giving maximal protection against dilution depended in a complex manner on several experimental variables; in general, however, 0.3–0.6 mg/ml was optimal.

The dependence of enzyme activity on acidic phospholipid was also shown by the fact that the cleavage activity of the undiluted extract was sensitive to adriamycin (Figure 3). This drug binds to acidic phospholipids (Goormaghtigh and Ruysschaert, 1984) and thereby inhibits the activity of several membrane enzymes (Cheneval *et al.*, 1985) as well as the unfolding of some precursor proteins by yeast mitochondria (Eilers *et al.*, 1989). Inhibition of the protease by adriamycin was relieved by addition of phosphatidyl serine or cardiolipin, further suggesting that the inhibition reflected the enzyme's requirement for acidic phospholipids.

Inner membrane protease I is absent in mutant pet ts2958

In order to prove that the cleavage of the cytb_2 intermediate measured by our *in vitro* assay indeed reflected the activity of the desired protease rather than that of some other enzyme, we showed that cleavage was absent in mutant *pet ts2858*. To this end, mutant *pet ts2858* was first crossed with the cytb_2-less strain whose mitochondria were normally used as a source of enzyme; dissection of the resulting four-spored asci showed that the ts phenotype and the cytb_2 deficiency each segregated independently 2:2, as expected. We then selected four tetrads which had one spore having only the cytb_2 deficiency, and another spore having both the cytb_2 deficiency and the ts marker. Finally, mitochondrial extracts from these two types of offspring were tested for cleavage of the cytb_2 intermediate. As shown in Figure 4, cleavage activity cosegregated with the wild-type allele of *pet ts2858*. This was confirmed by the absence of cleavage activity (not shown) in a strain whose *PET2858* gene (M.Behrens, G.Michaelis and E.Pratje, submitted) had been disrupted.

Solubilization of the inner membrane protease I

In order to study the association of the enzyme with yeast mitochondria, we extracted these with increasing concentrations of octyl-POE (Vestweber and Schatz, 1988). Extraction with 0.4% octyl-POE failed to solubilize any activity, even though it released most citrate synthase, a soluble matrix marker (Figure 5). Solubilization of the protease started at 0.5% octyl-POE; at 1% octyl-POE, ~60% of the protease activity was solubilized; under these conditions most cytochrome oxidase subunit IV, a marker of the inner membrane, remained sedimentable. Octyl-POE concentrations >1% caused loss of enzyme activity (not shown). Accordingly, we adopted a fractional solubilization protocol in which soluble mitochondrial proteins were first removed with 0.5% octyl-POE, whereupon much of the activity of the inner membrane protease I was solubilized, and separated from other membrane proteins, by 1% octyl-POE. The solubilized enzyme could be stored frozen at −70°C for several weeks without significant loss of activity; its specific activity was 2–3 times higher than that of solubilized mitochondria.

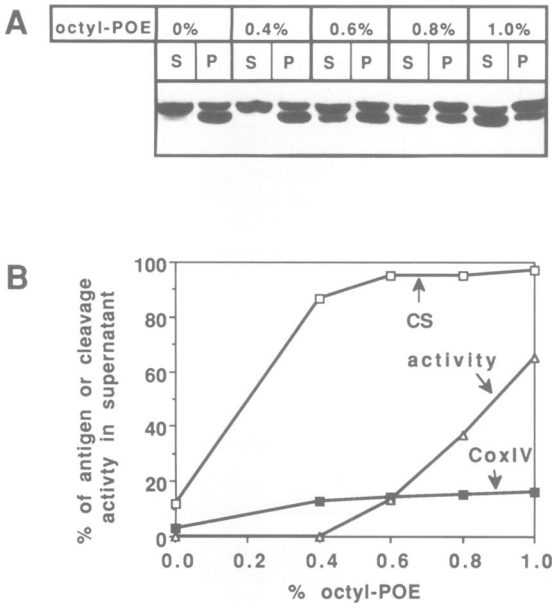

Fig. 5. Solubilization of the cleavage activity. Mitochondria (200 μg) of the cytb_2-deficient strain were incubated for 10 min at 0°C with the indicated concentrations [percentage (v/v)] of octyl-POE in 100 μl assay buffer. The mixture was centrifuged for 20 min at 30 p.s.i. in a Beckmann Airfuge. The pellet was resuspended in 100 μl of assay buffer and 40 μl of supernatant and resuspended pellet were assayed separately for cleavage of the cytb_2 intermediate (A; B 'activity'). In addition, pellet and supernatant were assayed for citrate synthase (CS; matrix marker) and cytochrome oxidase subunit IV (CoxIV, inner membrane marker) by quantitative immunoblotting.

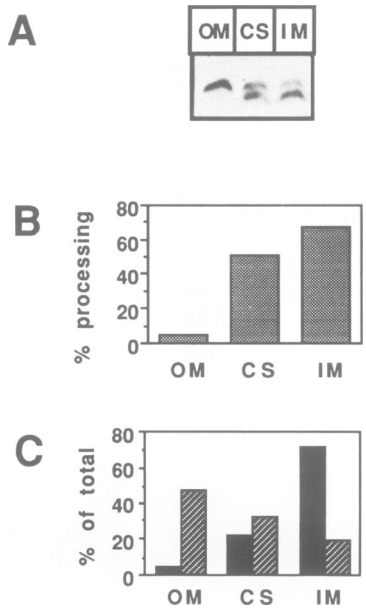

Fig. 6. The protease is associated with the mitochondrial inner membrane. **A.** Mitochondria were separated into outer membrane (OM), inner membrane (IM) and an intermediate density fraction enriched in membrane contact sites (CS). A 30 μg aliquot of each fraction was checked for cleavage of the cytb_2 intermediate. **B.** The gel in A was quantified by scanning. **C.** Each of the three submitochondrial fractions was assayed by immunoblotting for porin (outer membrane marker; hatched bars) and cytochrome oxidase subunit IV (inner membrane marker; filled bars).

Intramitochondrial localization of the protease

The solubilization studies described in the preceding section had shown that the protease is bound to one of the mitochondrial membranes. When mitochondria were subfractionated into outer membranes, inner membranes, and a fraction enriched in membrane contact sites (Pon *et al.*, 1989), the protease cofractionated with cytochrome oxidase subunit IV, an inner membrane enzyme (Figure 6). To check whether the enzyme was bound to the outer or the inner face of the inner membrane, we tested its accessibility to proteinase K in mitochondria and mitoplasts. To facilitate assay of the protease, these experiments were performed with mitochondria from the cytb_2-deficient strain (Figure 7). With intact mitochondria, the inner membrane protease I was completely resistant to proteinase K unless the mitochondrial membranes were disrupted with 1% octyl-POE. In mitoplasts, however, half of the enzyme was digested, even though all mitochondrial heat shock protein 70 (a soluble matrix marker) remained intact. Control experiments (not shown) revealed that at least 90% of the mitochondria had been converted to mitoplasts as evidenced by the release of cytochrome *c* peroxidase, a marker of the soluble intermembrane space. The inner membrane protease I is, thus, exposed on the outer face of the inner membrane. The antibody experiments described below support this conclusion.

A 21.4 kd protein correlates with the protease activity

The gene complementing the *ts2858* mutation can encode a 21.4 kd protein (M.Behrens, G.Michaelis and E.Pratje, submitted) and our present results show that this mutation abolishes the activity of the solubilized enzyme. The predicted 21.4 kd polypeptide may thus be a subunit of the protease. To test this possibility, we raised a rabbit antiserum against a chemically-synthesized peptide representing the C-terminal 12 residues of the predicted 21.4 kd protein. The antiserum was tested against the following four samples (Figure 8): an extract of *E.coli* cells that had been transformed with a plasmid encoding a fusion protein composed of β-galactosidase and the C-terminal half of the predicted 21.4 kd protein ('fus. prot.'); wild-type yeast mitochondria ('wild-type'); mitochondria from a yeast strain whose *PET2858* gene had been disrupted ('Δ*pet2858*'); and mitochondria from a yeast transformant overexpressing the *PET2858* gene ('overprod.'). As expected, the antiserum reacted strongly with the fusion protein. It also reacted faintly, but distinctly, with a 21.4 kd antigen in wild-type mitochondria. This signal was much stronger with mitochondria from the strain overproducing the *PET2858* gene and was absent from mitochondria of the *pet2858* null mutant (Figure 8A). All of these signals were eliminated by excess peptide antigen (Figure 8B). The signals in the 60–90 kd region appear to be unrelated to the 21.4 kd antigen as they were also seen with the *pet2858* null mutant and not competed away by excess peptide antigen.

These experiments suggested that the 21.4 kd polypeptide is a subunit of inner membrane protease I. Indeed, no cleavage activity was detected in mitochondrial extracts from a strain whose *PET2858* gene had been disrupted (Table I). On the other hand, overproduction of the 21.4 kd polypeptide in yeast cells did not increase the cleavage activity of mitochondrial extracts, even though it did increase the level of the 21.4 kd protein in these extracts 17.5-fold. The protease may thus contain at least one other subunit in

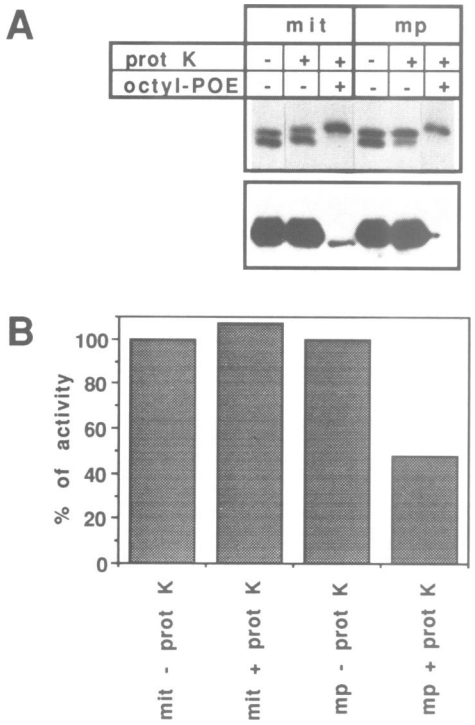

Fig. 7. The cleavage activity is localized on the outer face of the inner membrane. Mitochondria (mit) and mitoplasts (mp) (100 μg each) were treated with proteinase K (prot K) in the presence or absence of octyl-POE and then tested for cleavage of the cytb_2 intermediate. **A.** Upper panel: cleavage assay. Lower panel: the same fractions were analyzed by immunoblotting for the 70 kd mitochondrial heat shock protein (matrix marker). **B.** The data shown in the upper panel of A were quantified by scanning. Cleavage activity of samples not treated with proteinase K was set to 100%. The small amount of proteinase K resistant activity in samples digested in the presence of detergent (A, upper panel, lanes 3 and 6) was subtracted from the activity of each sample. In addition, cleavage activity of proteinase K treated mitoplasts was corrected for residual intact mitochondria (not shown). Each of these corrections amounted to ~10%.

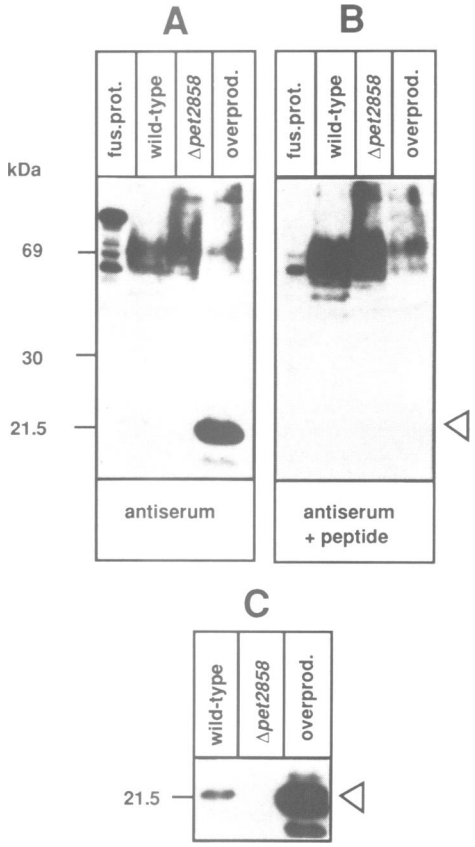

Fig. 8. Antiserum against an inner membrane protease I peptide recognizes a 21.4 kd protein in yeast mitochondria. Panels A, B and C show an autoradiograph of an immune blot with the following samples: a cell extract of an *E.coli* strain expressing a *lacZ–Pet2858* fusion protein ('fus. prot.'); wild-type yeast mitochondria ('wild-type'); mitochondria from a yeast strain whose chromosomal *PET2858* gene (encoding a subunit of inner membrane protease I) had been disrupted ('Δ*pet2858*'); and mitochondria from a yeast strain containing extra copies of the *PET2858* gene on a 2 μm-based multicopy plasmid and therefore overexpressing this gene ('overprod.'). Before electrophoresis and immunoblotting, all mitochondrial samples were washed with 0.5 M KP_i, pH 7.4; this was essential in order to remove a loosely bound cytosolic 35 kd protein that crossreacted with the antiserum. **A**, the blot was incubated with 200-fold diluted antiserum. **B**, same as A, but in the presence of 0.02 mg/ml peptide antigen. The autoradiographs shown in A and B were exposed for 4 h. **C**, the immune blot shown in A was exposed for 20 h in order to show clearly the absence of a 21.4 kd signal in the *pet2858* null mutant. The open arrowhead identifies the 21.4 kd *PET2858* gene product. The numbers on the left are mol. wt markers.

addition to the 21.4 kd polypeptide or it could misassemble upon overproduction.

The antiserum allowed us to confirm the submitochondrial localization of the enzyme by an immunological method. Rightside-out inner membrane vesicles (Hwang *et al.*, 1989) were incubated with different concentrations of immune IgGs with or without excess peptide antigen, reisolated by centrifugation, and subjected to the differential solubilization protocol outlined in a previous section. As shown in Figure 9, incubation of the vesicles with immune IgGs caused an 80% drop in the solubilized enzyme activity; no such drop was found with vesicles that had been incubated with the IgGs in the presence of excess peptide antigen.

This result confirms that the inner membrane protease I is exposed on the outer face of the inner membrane. It also shows that this exposed region includes the C-terminal part of the polypeptide. Finally, these data establish a direct link between the 21.4 kd open reading frame in the *PET2858* gene, the 21.4 kd antigen in yeast mitochondria, and the activity of the enzyme.

Based on this information, we used the antiserum to test whether the protease is an integral inner membrane protein. Mitochondria were extracted with buffer at pH 11.5 (Fujiki *et al.*, 1982) and the soluble extract as well as the insoluble pellet were assayed for the 21.4 kd protein and two marker proteins by immunoblotting (Figure 10). The 21.4 kd protein, like the integral inner membrane protein cytochrome c_1, remained insoluble, whereas cytochrome *c* (a peripheral inner membrane protein) was completely solubilized. This result, together with the data described in earlier sections, establishes the enzyme as an integral protein of the inner membrane.

Discussion

Mitochondrial sorting proteases

The proteases removing the sorting sequences from proteins translocated into the mitochondrial intermembrane space have so far received little attention. There was evidence to

Table I. Overproduction of the *PET2858*-encoded 21.4 kd polypeptide does not increase the activity of mitochondrial inner membrane protease I

	Specific activity of inner membrane protease I (U/mg)	21.4 kd antigen (arbitrary units)
Δb_2	0.0156	(1)
Δb_2 + *PET2858*	0.0160	17.5
Δpet2858	<0.001	<0.02

The cytb_2-deficient strain Δb_2 was transformed with the 2 μm plasmid pFM2-1−*PET2858* containing the *PET2858* gene (Δb_2 + *PET2858*), or with the unrelated insert-free 2 μm plasmid pVT-101 (Δb_2). The transformants were grown overnight at 30°C under selective conditions in uracil-free synthetic medium. To promote optimal mitochondrial development, the cells were transferred for 5 h at 40°C to 1% Bacto-yeast extract, 1% Bacto-peptone, 2% galactose. Strain Δpet2858 carrying a disrupted inner membrane protease I gene was grown for ~16 h on YP-galactose. Mitochondria were prepared (Daum *et al.*, 1982) and checked for cleavage activity under standard assay conditions, and for the presence of the 21.4 kd antigen by immunoblotting.

suggest that yeast mitochondria contain at least two such proteases differing in precursor specificity, but neither the intramitochondrial locations nor the properties of any of these enzymes were known.

An assay for inner membrane protease I

In this study we have worked out an assay for the protease which removes the sorting sequence from the cytb_2 intermediate and the signal sequence from cytochrome oxidase subunit II. With the aid of this assay we have been able to show that the enzyme is firmly bound to the outer face of the inner membrane, that it contains a 21.4 kd subunit, that it may be a hetero-oligomer, and that it requires acidic phospholipid for activity. In view of these features, we term this enzyme 'inner membrane protease I'.

The main difficulty in developing this assay was to present the substrate (the cytb_2 intermediate) in the correct conformation for cleavage to occur. In contrast to the matrix-localized MAS-protease, inner membrane protease I was inactive towards precursors which had been synthesized in a reticulocyte lysate, even when the intermediate form of cytb_2 was produced *in vitro*. However, it did act upon the cytb_2 intermediate in detergent-solubilized mitochondria from yeast mutant *pet ts2858*. In this mutant, the 21.4 kd subunit of the protease is defective, maturation of cytb_2 is interrupted after the first cleavage step, and the cells accumulate cytb_2 intermediate bound to the outer face of the inner membrane (Pratje and Guiard, 1986). This intermediate is extractable at pH 11.5 (not shown). Thus, it is probably still stuck in the import pathway, in the proper conformation for cleavage by its cognate protease. Although this explanation is tentative, it would be in line with the previous observation that yeast pre-pro-α factor requires the presence of SDS in order to be cleaved by yeast signal peptidase (YaDeau and Blobel, 1989). Similarly, proteolytic processing of preplacental lactogen by mammalian signal peptidase is stimulated by an antibody against this precursor protein (Lively and Walsh, 1981). The detergent or the antibody might be expected to favor a conformation of the precursor which facilitates cleavage by the corresponding signal peptidase.

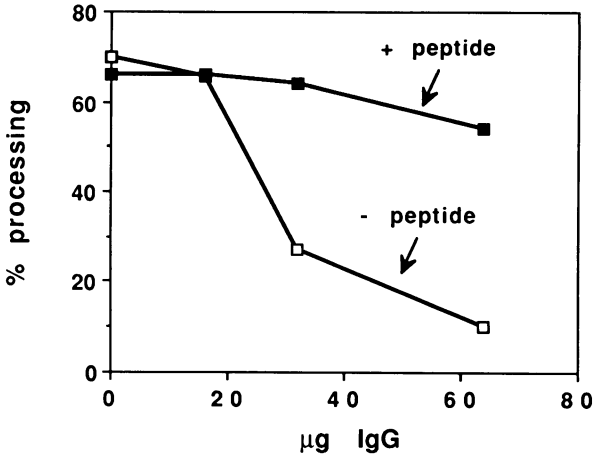

Fig. 9. Pretreatment of inner membrane vesicles with anti-inner membrane protease I IgGs inhibits the cleavage activity. Inner membrane vesicles (30 μg) were incubated for 5 h at 0°C with the indicated concentrations of anti-inner membrane protease I IgGs in the absence and presence of the inner membrane protease I peptide, reisolated by centrifugation, and tested for cleavage of the cytb_2 intermediate. Cleavage was quantified by scanning. Immunoblot analysis of the vesicles (not shown, but see Figure 8) confirmed that the only antigen specifically recognized by the antiserum was the 21.4 kd *PET2858* gene product.

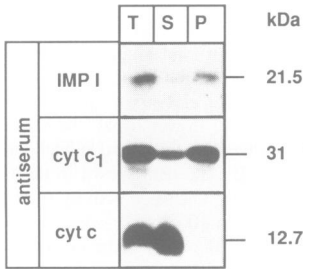

Fig. 10. Inner membrane protease I is an integral membrane protein. Mitochondrial membranes were prepared as described by Vestweber *et al.* (1988) and extracted at pH 11.5. Total membranes (T), pH 11.5 extract (S) and pH 11.5 pellet (P) (each corresponding to 200 μg of mitochondria) were analyzed by SDS−14% PAGE and immunoblotting for the inner membrane protease I polypeptide, cytochrome c_1 (an integral membrane protein) and cytochrome *c* (a peripheral inner membrane protein). The figure shows the mol. wt region of the corresponding antigen; mol. wts are indicated on the right.

Relationship to other signal peptidase

The similarity between inner membrane protease I and *E.coli* leader peptidase is perhaps one of the most interesting results emerging from our studies. Both enzymes are integral membrane proteins, both require acidic phospholipid for activity (de Vrije *et al.*, 1988), and both have their catalytic site and C-terminal part exposed on the *trans*-side of the membrane barrier. The two enzymes also have significant sequence identity (M.Behrens, G.Michaelis and E.Pratje, submitted). This relationship to prokaryotic leader peptidase is in harmony with the proposal that sorting of proteins to the mitochondrial intermembrane space is functionally analogous to the export of bacterial proteins to the periplasm (Hartl *et al.*, 1987; Hartl and Neupert, 1990). This homology also reinforces the view that the 21.4 kd protein is a subunit of the protease, and not merely a chaperone-like ancillary

protein required for cleavage of a few select precursors. On the other hand, inner membrane protease I differs from *E.coli* leader peptidase in that it appears to contain more than one type of subunit; also its 21.4 kd subunit is distinctly smaller than the single subunit (36 kd) of *E.coli* leader peptidase. In these respects, inner membrane protease I is more similar to the signal peptidase associated with the endoplasmic reticulum. Yet another related enzyme appears to be the thylakoid-associated protease that removes the sorting sequence from precursors imported into the thylakoid lumen of chloroplasts (Hageman *et al.*, 1986). This enzyme, like inner membrane protease I, is membrane-bound and resembles bacterial leader peptidase in its ability to cleave bacterial precursor proteins correctly (Halpin *et al.*, 1989). It has not yet been purified to homogeneity and neither its gene(s) nor its subunit composition are known.

Other mitochondrial sorting proteases

Cytochrome c_1 and cytochrome *c* peroxidase are two additional proteins that are cleaved twice during their voyage to the intermembrane space (Attardi and Schatz, 1988; Hartl and Neupert, 1990). Their targeting signals resemble that of cytb_2 in that they contain an N-terminal matrix-targeting signal followed by a hydrophobic sequence which, in the case of cytochrome c_1, has been shown to act as a sorting signal for the intermembrane space (van Loon *et al.*, 1986, 1987; Van Loon and Schatz, 1987). As a *pet2858* null mutant accumulates the correctly processed forms of cytochrome c_1 and cytochrome *c* peroxidase (M.Behrens, G.Michaelis and E.Pratje, submitted), maturation of these proteins is probably mediated by yet another protease (or proteases) whose identity and properties are unknown. The protease cleaving the cytochrome c_1 intermediate, like inner membrane protease I, appears to be sensitive to the conformation of its substrate, as cleavage requires the attachment of heme to the intermediate form of apocytochrome c_1 (Ohashi *et al.*, 1982; Nicholson *et al.*, 1989). Further work may well uncover additional proteases that participate in the maturation of precursors transported to the different mitochondrial compartments.

Materials and methods

Strains and plasmids used

The wild-type *Saccharomyces cerevisiae* strain SC167 (*Matα, ade1*) and the mutant *pet ts2858* (*Matα, ade1, pet ts2858*) are described by Michaelis *et al.* (1982). *S.cerevisiae* BSL 1 (*Matα, lys2, ura3, leu2-3, leu2-112, his4-519*) (Bibus *et al.*, 1988) was used for disrupting the gene coding for cytb_2 as follows: the *LEU2* gene was inserted at the *Bam*HI site of the cytb_2 gene; the resulting cytb_2–*LEU2* DNA fragment was cut out with *Spe*I and *Bgl*II and used to transform BSL 1 to leucine independence. Leucine prototrophs were checked for the absence of cytb_2 by immunoblotting. This yielded strain Δb_2. Crossing this strain to *pet ts2858* resulted in spores with the phenotype (ts/Δb_2) and (wt/Δb_2) which were used to show absence of cleavage activity in the ts mutant. The yeast strain disrupted for inner membrane protease I (Δ*pet2858*) derived from wild-type strain KN79 (*Matα, leu2-1, trp1-1*) is described (M.Behrens, G.Michaelis and E.Pratje, submitted). To study the effect of overproduction of the 21.4 kd subunit, we transformed Δb_2 with plasmid pFM2-1-*PET2858* (M.Behrens, G.Michaelis and E.Pratje, submitted). The β-galactosidase fusion protein containing the carboxy-terminal half of inner membrane protease I was constructed by inserting the *Bam*HI–*Sna*BI fragment of the *PET2858* gene into the *Bam*HI, *Xba*I (filled in with Klenow enzyme) sites of plasmid pUEX3 (Bressan and Stanley, 1987).

In vitro assay for inner membrane protease I activity

Preparation of mitochondria containing the cytb_2 intermediate. The yeast strain *pet ts2858* was grown as described on 10% glucose at 23°C with subsequent incubation for 12 h at 37°C in phosphate buffer containing DL-lactate (Pratje and Guiard, 1986). Mitochondria were isolated (Daum *et al.*, 1982), resuspended at a protein concentration of 6 mg/ml in 20 mM Tris–Cl, pH 7.5, 0.6 M sorbitol, 10 mM NEM, 1 mM phenylmethanesulfonylfluoride (PMSF) and incubated for 10 min on ice. NEM was then quenched by adding dithiothreitol to 20 mM. The mitochondrial suspension was frozen in liquid nitrogen and stored in aliquots at −70°C.

Source of enzyme. Strain Δb_2 was grown in 1% Bacto-yeast extract, 1% Bacto-peptone, 2% galactose and mitochondria were prepared (Daum *et al.*, 1982).

Preparation of phosphatidyl serine liposomes. A volume corresponding to 10 mg of the desired phospholipid (supplied in chloroform or chloroform: methanol by Sigma Chemical Co.) was dried down under a stream of nitrogen. The dried residue was suspended in 1 ml of 20 mM Tris–Cl, pH 7.5, 0.5 mM EDTA and the suspension was sonicated five times for 1 min in a bath-type sonifier (Transsonic T400, ELMA Co.) containing ice-cold water. The resulting suspension was frozen in 0.2 ml aliquots in liquid nitrogen and stored at −70°C. Just before use, each aliquot was thawed and sonicated again for 15 s.

The standard assay. Twenty μl of NEM-treated mitochondria from mutant *pet ts2858* (120 μg protein; containing 0.1–0.5 μg of cytb_2 intermediate; see preceding section) were mixed with the desired amount of enzyme (derived from cytb_2-deficient mitochondria) and adjusted to a final volume of 0.1 ml and the following final concentrations: Tris–Cl, pH 7.5, 20 mM; $MgCl_2$, 10 mM; octyl-POE, 0.4% (v/v); PMSF, 1 mM. Phosphatidyl serine liposomes were then added to 0.3–0.6 mg/ml (depending on the fraction tested; see Results) and the mixture was gently agitated on a Vortex mixer, followed by a 15 s sonication in the bath-type sonicator at room temperature. After incubation for 1 h at 30°C, the reaction was stopped by adding 0.1 ml of 3-fold concentrated SDS sample buffer and heating the mixture to 100°C for 3 min. Finally, 70 μl of the sample was analyzed by SDS–10% PAGE and immunoblotting with antiserum against cytb_2. To obtain optimal separation of the cytb_2 intermediate from mature cytb_2, electrophoresis was continued for 1 h after the tracking dye had electrophoresed off the gel.

Solubilization of inner membrane protease I

Two hundred micrograms of cytb_2-deficient mitochondria were suspended in 100 μl 20 mM Tris–Cl, pH 7.5, 1 mM PMSF, containing 0, 0.4, 0.6, 0.8 or 1.0% (v/v) octyl-POE. After 10 min at 0°C, the samples were centrifuged in an Airfuge (Beckmann Instruments, Inc.) for 20 min at 4°C at 30 p.s.i. (100 000 *g*). The supernatants were collected and adjusted to 1.0% octyl-POE. The pellets were resuspended by sonication in the bath-type sonicator in 100 μl of 20 mM Tris–Cl, pH 7.5, 1 mM PMSF containing 1% octyl-POE. Forty μl of each sample was either assayed for cleavage activity, or checked by immunoblotting for matrix and membrane markers. These exploratory experiments led to the following standard solubilization procedure: mitochondria from the cytb_2-deficient strain were suspended to 2 mg/ml in 20 mM Tris–Cl, pH 7.5, 1 mM PMSF, 0.5% octyl-POE and centrifuged for 30 min at 4°C at 100 000 *g*. The supernatant was discarded and the pellet was resuspended by sonication in the bath-type sonicator in one-fifth of the original volume of 20 mM Tris–Cl, pH 7.5, 1 mM PMSF, 1% octyl-POE. The resulting suspension was centrifuged for 90 min at 4°C at 100 000 *g*. The final supernatant always contained at least 50% of the enzyme activity present in the unfractionated octyl-POE extract.

Treatment of mitochondria or mitoplasts with proteinase K

Mitochondria or mitoplasts (300 μg protein each) from cytb_2-deficient cells were suspended in 0.3 ml of 20 mM Tris–Cl, pH 7.5, 0.6 M sorbitol and divided into three equal samples. One was incubated for 15 min at 0°C with 0.1 mg/ml proteinase K, the second with 0.1 mg/ml proteinase K and 1% octyl-POE, and the third without further additions. After adding 1 mM PMSF to all samples, the first and the third samples were adjusted to 1% octyl-POE. A 40 μl aliquot of each sample was tested for cleavage of cytb_2 intermediate in the standard assay. The cleavage reaction was terminated by adding trichloroacetic acid to 5% and heating the sample for 5 min at 60°C. The denatured proteins were suspended in 3-fold concentrated SDS sample buffer containing 1 mM PMSF and heated for 3 min at 100°C. This treatment completely inactivated any residual traces of proteinase K which could interfere with the analysis (B.Glick, in preparation). Another 40 μl aliquot was assayed by immunoblotting for mitochondrial heat shock protein 70, a marker of the soluble matrix space (Scherer *et al.*, 1990).

Inhibition of the cleavage activity by IgGs specific for inner membrane protease I

Rightside-out inner membrane vesicles from wild-type mitochondria were prepared by sonication and sucrose gradient centrifugation (Pon *et al.*, 1989; Hwang *et al.*, 1989). Immunoblotting showed that all the mature cytb_2 was removed during the procedure. These vesicles could thus be used in the *in vitro* cleavage assay. IgGs from the antipeptide antiserum were prepared by chromatography on protein A–Sepharose (Hines *et al.*, 1990) and concentrated to 150 mg/ml using the Centricon system (Amicon Co., USA). Inner membrane vesicles (60 μg in 120 μl) were incubated with different concentrations of IgGs in the absence and presence of 0.02 mg/ml of the peptide antigen. After 5 h at 4°C, the vesicles were pelleted in a Beckmann Airfuge (20 min at 4°C at 30 p.s.i.). The supernatant was discarded and the pellet resuspended by sonication in 75 μl of 20 mM Tris–Cl, pH 7.5. The substrate, $MgCl_2$, octyl-POE and phosphatidyl serine were added and the assay was performed under standard conditions. Control experiments (not shown) revealed that reisolation of the vesicles in the Airfuge removed essentially all of the unbound IgGs.

Extraction of mitochondrial membranes at pH 11.5

Mitochondria (2 mg/ml protein) were extracted with 0.5% octyl-POE (Vestweber and Schatz, 1988), resuspended to 2 mg/ml in 0.1 M Na_2CO_2, pH 11.5, sonicated in the bath-type sonicator for 30 s at room temperature and centrifuged for 1 h at 4°C at 100 000 *g*. The supernatant was saved and the pellet was resuspended in the original volume of Na_2CO_2. The starting membranes, the supernatant and the resuspended pellet were precipitated wth 5% trichloroacetic acid and an aliquot (equivalent to 200 μg of mitochondria) was analyzed by SDS–14% PAGE and immunoblotting with the antipeptide antiserum and antisera against two marker proteins.

Miscellaneous

Published methods were used for SDS–PAGE (Hurt *et al.*, 1984), immunoblotting (Haid and Suissa, 1983), production of antisera (Suissa and Reid, 1983), preparation of mitoplasts (Daum *et al.*, 1982), affinity purification of antisera on nitrocellulose strips (Vestweber *et al.*, 1989), subcellular fractionation (Hase *et al.*, 1984) and protein determination (BCA method; company brochure published by Pierce Chemical Co.). A peptide corresponding to the C terminus of inner membrane protease I (INNTFLDVQAKSD) was synthesized and coupled to hemocyanin by Multiple Peptide Systems Co., San Diego, USA. This peptide was used as an antigen to produce mitochondrial inner membrane I specific antibodies in rabbits.

Quantification of autoradiographs was done on a 300 A Computing Densitometer (Molecular Dynamics Co.). Octyl-POE was kindly donated by Dr Juerg Rosenbusch (Biocenter, University of Basel).

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