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A yeast mutant temperature-sensitive for mitochondrial assembly is deficient in a mitochondrial protease activity that cleaves imported precursor polypeptides

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We have previously described two yeast mutants which, at elevated temperature, stop growing and accumulate precursors to several imported mitochondrial proteins. We now show that one of these mutants (*mas 1*) is deficient in a matrix-located protease activity which cleaves the pre-sequences from mitochondrial precursor proteins. Isolated *mas 1* mitochondria catalyze oxidative phosphorylation, exhibit respiratory control and import mitochondrial precursor polypeptides, but are defective in removing transient pre-sequences from imported precursors. The phenotype of the *mas 1* mutant suggests that the matrix-located processing protease is essential for growth and for mitochondrial assembly.

Key words: yeast/*mas 1* mutant/protease/pre-sequence/mitochondria

Introduction

The formation of a mitochondrion requires the import of hundreds of different polypeptides from the cytosol and the sorting of these proteins into their correct intramitochondrial compartments (Schatz and Butow, 1983; Hay *et al.*, 1984). Some features of this import process have been identified by studying the uptake of *in vitro*-synthesized, radiolabeled polypeptides by isolated mitochondria. Proteins destined for internal mitochondrial compartments (matrix, inner membrane and intermembrane space) are generally made as precursors with transient amino-terminal pre-sequences (Hay *et al.*, 1984). These pre-sequences carry the information for targeting the precursor to the mitochondrion (Hurt *et al.*, 1984; Horwich *et al.*, 1985). The precursors bind to the mitochondrial surface and then translocate across the mitochondrial membranes by an unknown mechanism which requires an electrochemical potential across the mitochondrial inner membrane. Finally, the pre-sequence is proteolytically removed in the matrix space.

Little is known about the molecules that mediate import and intramitochondrial sorting of mitochondrial proteins. The first component to be identified was a matrix-localized processing protease (Böhni *et al.*,1980; Miura *et al.*, 1982). This enzyme, purified ~100-fold from yeast mitochondria (McAda and Douglas, 1982; Böhni *et al.*, 1983) specifically cleaved several mitochondrial precursor polypeptides; the precursor to cytochrome oxidase subunit V was shown to be cleaved at the correct position (Cerletti *et al.*, 1983). This protease was inhibited by metal chelators, could be reactivated by Co^{2+} or Zn^{2+} and only cleaved native mitochondrial precursor polypeptides. Since this protease was still present in cytoplasmic petite mutants lacking mitochondrial protein synthesis, it must be itself imported into mitochondria (Böhni *et al.*, 1983). The enzyme has not yet been purified to homogeneity nor has the enzymic activity been assigned to a specific polypeptide in the partially purified preparations; it is, thus, still unclear whether all mitochondrial pre-sequences are removed by this protease and whether the protease is made as a larger precursor and cleaves itself during import. Also, the role of the protease in the import pathway remains ill-defined. Import of a precursor into mitochondria is not always tightly linked to proteolytic cleavage; under special conditions, pulse-labeled precursors can be chased into mitochondria without being cleaved (Reid *et al.*, 1982; Zwizinski and Neupert, 1983; Hurt *et al.*, 1985). However, correct processing of imported precursors may well be necessary for sustained import into mitochondria.

In an attempt to dissect the mitochondrial import machinery we have isolated two temperature-sensitive yeast mutants which accumulate mitochondiral precursor polypeptides at the non-permissive temperature, 37°C. These mutants are also temperature-sensitive for growth, but only after a lag of 2–3 generations. Since defective maturation of mitochondrial precursor polypeptides should interfere with *m*itochondrial *as*sembly, we termed these mutants *mas* 1 and *mas* 2 (Yaffe and Schatz, 1984).

Here we report that mitochondria isolated from the *mas 1* mutant have drastically lowered activity of the matrix-localized processing protease. Further work will be necessary to establish whether the *mas 1* locus codes for a subunit of the protease or for some regulatory element. Nevertheless, this mutant offers new possibilities for defining the role of proteolytic processing in mitochondrial protein import.

Results

Isolated mas 1 mitochondria import mitochondrial precursors, but are defective in processing them

When isolated, energized wild-type mitochondria were incubated at 23°C with ^{35}S-labeled precursor to cytochrome *c* oxidase subunit IV for 2, 5 or 10 min, they converted part of the precursor to mature subunit IV (Figure 1, lanes 1–3) which was inaccessible to externally added trypsin (lanes 7–9). Residual precursor associated with the mitochondria was completely digested by externally added trypsin and, thus, presumably bound to the mitochondrial surface. This result is consistent with earlier work (Hurt *et al.*, 1984a). In contrast, *mas 1* mitochondria accumulated both mature and precursor form of subunit IV in a trypsin-inaccessible form (lanes 4–6 and 10–12). When the mitochondrial membranes were disrupted by 1% Triton X-100, all labeled polypeptides were degraded by trypsin (not shown; but see Figure 5A of Hurt *et al.*, 1984a). This indicates that not only mature radiolabeled subunit IV but also some amount of the subunit IV precursor is located inside the *mas 1* mitochondria. The rate of import by *mas 1* mitochondria (as measured by the radioactivity present in the imported precursor plus the imported mature subunit IV) was 60–77% the rate of import by wild-type mitochondria (as measured by the radioactivity present in the imported mature subunit). A similar result was observ-

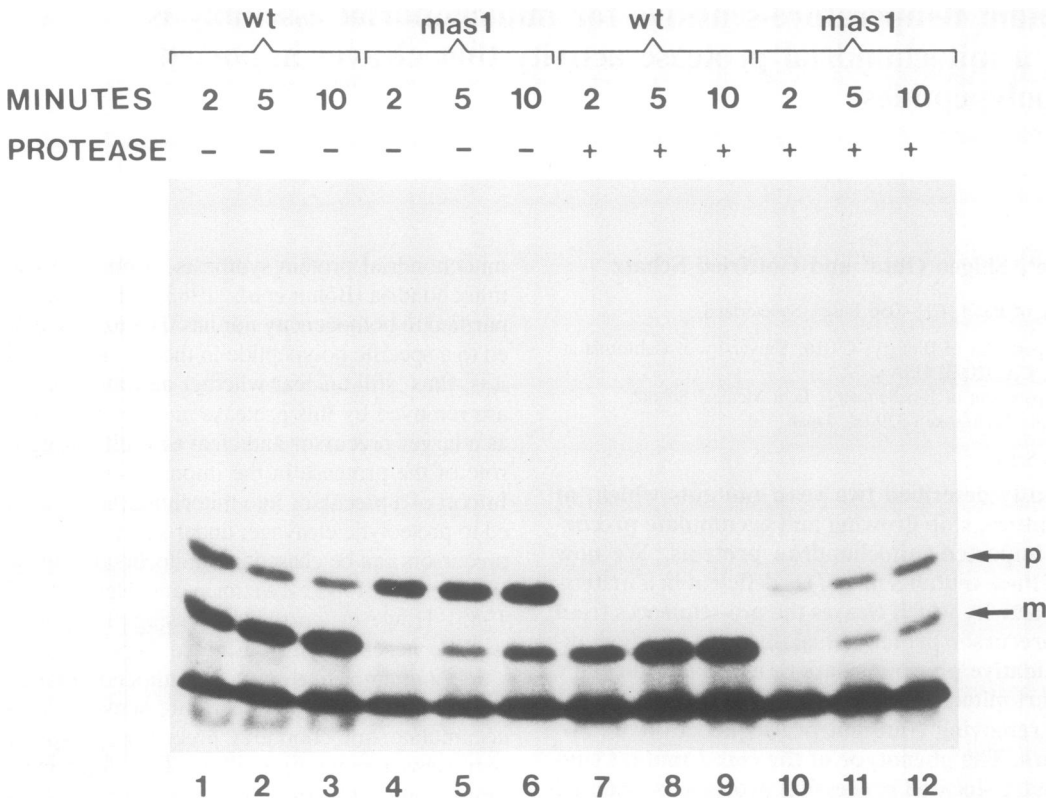

Fig. 1. Mitochondria from the *mas 1* mutant are defective in processing imported precursor to cytochrome *c* oxidase subunit IV. Isolated mitochondria from wild-type (wt) or *mas 1* cells were incubated with precursor to cytochrome oxidase subunit IV which had been synthesized and labeled with [^{35}S]methionine *in vitro* (Hurt *et al.*, 1984a). The import mixture (200 μl) consisted of 200 μg mitochondria, 15 μl reticulocyte lysate containing the labeled precursor, 1 mM ATP, 1 mM $MgCl_2$, 5 mM P-enolpyruvate, 2 units pyruvate kinase, 40 mM KCl, 5 mM GTP, 5 mM methionine, 0.6 M mannitol and 20 mM Hepes-KOH, pH 7.4. Samples were incubated for the indicated times at 23°C and then chilled rapidly to 0°C. Some samples were incubated further at 0°C with 250 μg/ml trypsin for 20 min. Trypsin was then inhibited by addition of trypsin inhibitor and phenyl methyl sulfonyl fluoride to concentrations of 2.5 mg/ml and 1 mM, respectively; the incubation mixtures were layered on 1.0 ml cushions of 25% sucrose in 20 mM Hepes-KOH, pH 7.4 at 0°C, and centrifuged in an Eppendorf centrifuge (Model 3400) for 10 min at 4°C. The mitochondrial pellet was taken up in 50 μl SDS-sample buffer and heated at 95°C for 4 min. Proteins were resolved on a 13% polyacrylamide gel run in the presence of SDS. Following electrophoresis the gel was dried and fluorographed (p = precursor; m = mature subunit IV). In this and all other experiments, the binding of precursors to the mitochondrial surface was not characterized; at least some of this binding may well be non-specific.

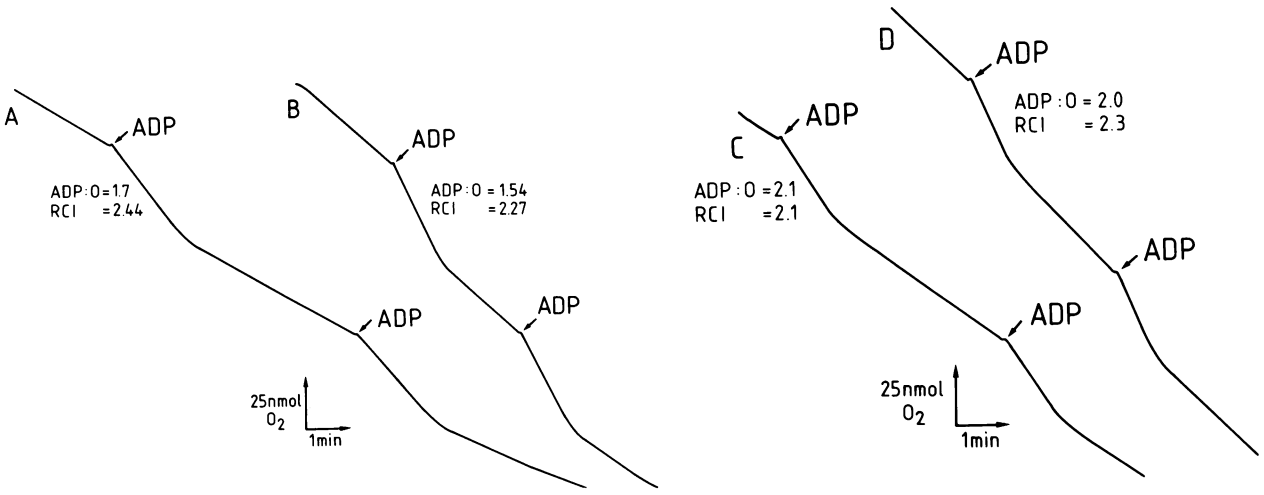

Fig. 2. Mitochondria from the *mas 1* mutant catalyze oxidative phosphorylation. Mitochondria were prepared from wild-type cells (**A,C**) and the *mas 1* mutant (**B,D**) as described by Daum *et al.* (1982), but with the following modifications: (i) cells were grown at 23°C on semisynthetic medium containing 2% galactose, 0.1% glucose; (ii) cells were harvested at A_{600} = 3−4; (iii) incubation with dithiothreitol was for 25 min at 23°C; (iv) treatment with Zymolyase 20 000 (2.5 mg/g wet weight of cells) was for 60−90 min at 23°C. The isolated mitochondria (250 μg protein) were analyzed at 23°C in a 0.8 ml thermostatted polarographic reaction chamber fitted with a Clark-type oxygen electrode. The assay medium was 20 mM Hepes-KOH pH 7.4, 0.6 M mannitol, 10 mM potassium phosphate, 2 mM $MgCl_2$, 1 mM EDTA, 5 mg bovine serum albumin/ml and either 10 mM Tris-succinate (**A,B**) or 10 mM Tris-malate plus 10 mM Tris-pyruvate (**C,D**). Where indicated, 5 μl of 17 mM ADP (pH 7.4) was added. RCI=respiratory control index (rate of respiration plus ADP rate of respiration after exhaustion of added ADP).

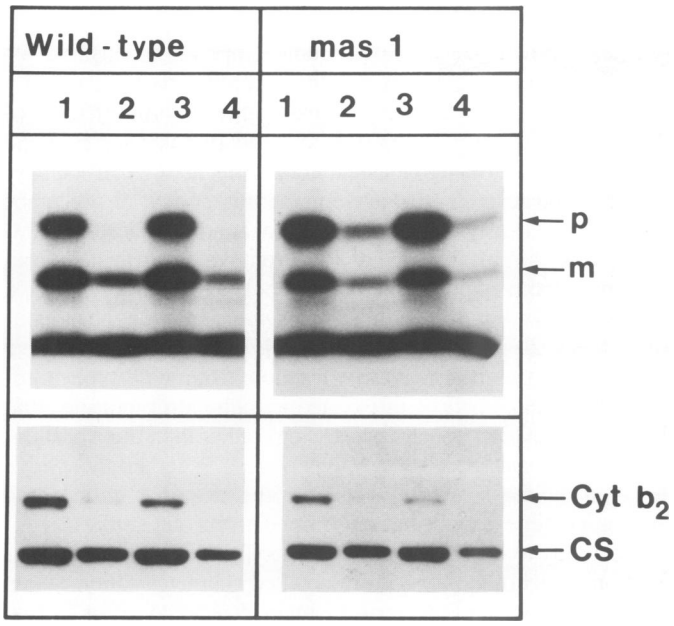

Fig. 3. The unprocessed subunit IV precursor imported by energized *mas 1* mitochondria is located inside the inner membrane. Import of cytochrome oxidase subunit IV precursor into mitochondria isolated from wild-type and *mas 1* mutant cells was carried out as described in Figure 1. Incubations were for 10 min at 23°C. The samples were then chilled at 0°C and centrifuged through a cushion of 25% sucrose in 20 mM Hepes-KOH, pH 7.4 as described in Figure 1. Mitochondrial pellets were resuspended in 200 μl of either 0.6 M mannitol, 20 mM Hepes-KOH, pH 7.4 (**lanes 1,2**) or 0.1 M mannitol, 20 mM Hepes-KOH, pH 7.4 (for mitoplast preparation, **lanes 3,4**). All samples were held on ice for 20 min, after which 15 μl unlabeled reticulocyte lysate in either 0.6 M mannitol or 0.1 M mannitol was added to each sample. Samples in **lanes 2** and **4** were treated with trypsin, and the trypsin was subsequently inhibited as described in Figure 1. All samples were then spun for 10 min in an Eppendorf centrifuge at 4°C. The pellets were taken up in SDS-sample buffer. **Upper panel:** samples were analyzed for imported radiolabeled subunit IV as described in Figure 1. p and m, precursor and mature form of cytochrome *c* oxidase subunit IV. **Lower panel:** samples were subjected to immune blotting with antisera against cytochrome b_2 (Cyt b_2; a marker for the intermembrane space) and citrate synthase (CS; a marker for the matrix space). The percentage of protected subunit IV precursor in this experiment appears to be lower than in Figure 1. This difference, however, is only a reflection of the high amount of precursor associated with the surface of *mas 1* mitochondria in the experiment of Figure 3. The nature of this association was not characterized; at least some of it may be non-specific.

ed by studying import of purified, radioiodinated precursor of the F_1-ATPase β-subunit: upon incubation with energized *mas 1* mitochondria, both the precursor and the mature form became inaccessible to externally-added trypsin (not shown).

When assayed at 37°C, import by either *mas 1* or wild-type mitochondria was greatly reduced, probably because the mitochondria become unstable. However, since isolated *mas 1* mitochondria exhibited a distinctly abnormal phenotype if assayed for protein import, even at 23°C, all further comparisons between these two types of mitochondria were done at 23°C. We conclude that the *mas 1* mutation does not block import of precursors into mitochondria, but the intramitochondrial processing of these precursors.

Isolated mas 1 mitochondria are well coupled

The *mas 1* mutation does not merely labilize mitochondria or interfere with mitochondrial energy coupling: mitochondria isolated from the *mas 1* mutant readily oxidized succinate or pyruvate-malate, exhibited good respiratory control and phosphorylated ADP with high efficiency (Figure 2). All measured values were not significantly different from those of the corresponding wild-type mitochondria. The experiments shown in Figure 2 were performed at 23°C; at higher temperatures, both *mas 1* and wild-type mitochondria rapidly lost respiratory control (not shown). Since the *mas 1* phenotype was already manifest at 23°C with isolated mitochondria (Figure 1) it does not result from a defect in oxidative phosphorylation or a general labilization of the mitochondrial organelles.

The unprocessed subunit IV precursor imported by isolated mas 1 mitochondria is located inside the inner membrane

Much of the unprocessed subunit IV precursor imported by *mas 1* mitochondria is inaccessible to externally-added trypsin not only in mitochondria (Figure 3, upper panel; compare lanes 1 and 2) but also in mitoplasts (i.e., osmotically shocked mitochondria whose outer membrane had been disrupted; Figure 3, upper panel; compare lanes 3 and 4). The immune blots (Figure 3, lower panel) show that, in this particular experiment, the 'mitochondria' had already lost most of their outer membrane barrier since cytochorme b_2 was largely susceptible to externally added trypsin. However, the inner membrane barrier was largely intact in mitochondria as well as in mitoplasts since the matrix enzyme citrate synthase was largely trypsin-inaccessible. Taken together, the two panels of Figure 3 show clearly that both the mature and precursor form of subunit IV are inaccessible to external trypsin under conditions where all of the mitochondrial cytochrome b_2 had been removed by a combination of osmotic shock and external trypsin. The unprocessed imported precursor is, thus, located inside the inner membrane. In principle, the unprocessed precursor could also be bound to the outside of the inner membrane in a trypsin-resistant form, but we consider this less likely. The analogous results obtained with wild-type mitochondria confirm those published earlier (Hurt *et al.*, 1984a, 1984b) and are included for comparison. We conclude that *mas 1* mitochondria transport the subunit IV precursor to the matrix, but process it only very slowly.

A non-cleavable 'precursor protein' is imported by mas 1 mitochondria as efficiently as by wild-type mitochondria

The results of Figures 1 and 3 suggested that *mas 1* mitochondria were not defective in importing precursors, but only in processing them. *mas 1* mitochondria should thus become indistinguishable from wild-type mitochondria if import experiments are carried out with a precursor containing a non-cleavable leader sequence. This was indeed the case (Figure 4). The non-cleavable 'precursor' used in this experiment was a fusion protein containing 16 amino-terminal residues from the subunit IV pre-sequence fused to the amino terminus of mouse dihydrofolate reductase. Isolated wild-type mitochondria import this fusion protein, but fail to process it to a smaller form (Hurt *et al.*, 1985). Figure 4 shows that *mas 1* mitochondria import this fusion protein at roughly the same rate as do wild-type mitochondria. This result further supports the view that the *mas 1* mutation does not interfere with the translocation of precursors across the two mitochondrial membranes.

A soluble matrix fraction isolated from mas 1 mitochondria is deficient in a chelator-sensitive protease activity that cleaves mitochondrial precursor polypeptides

The import experiments with isolated *mas 1* mitochondria suggested that the *mas 1* mutation interfered with the proteolytic removal of pre-sequences from imported precursor polypeptides. To test this directly, soluble matrix fractions were prepared from wild-type and *mas 1* mitochondria and assayed for their ability to process mitochondrial precursor polypeptides (Figure 5). In

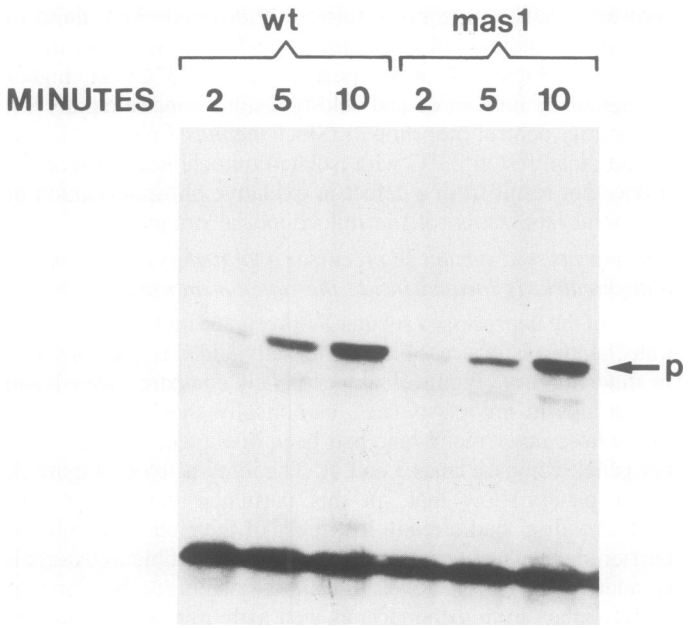

Fig. 4. A non-cleavable precursor protein is imported at normal rates by *mas 1* mitochondria. Isolated mitochondria from wild-type (wt) or *mas 1* cells were allowed to import a fusion protein containing the 16 amino-terminal amino acids from the subunit IV pre-sequence fused to mouse cytosolic dihydrofolate reductase (Hurt *et al.*, 1985). This fusion protein had been synthesized and labeled *in vitro*. Samples were then incubated with 250 μg/ml proteinase K at 0°C for 30 min and the protease was inhibited by addition of phenyl methyl sulfonyl fluoride to 1 mM. The mitochondria were recovered, and proteins extracted and analyzed as described in Figure 1. p = uncleaved fusion protein ('precursor').

line with earlier work, a soluble matrix fraction from wild-type mitochondria processed a fusion protein consisting of 53 amino-terminal residues from the subunit IV precursor attached to the amino terminus of mouse dihydrofolate reductase (Hurt *et al.*, 1984a). Processing was rapid at either 0° or 23°C being almost maximal after 5 min at 23°C or 15 min at 0°C (Figure 5A). (A variable fraction of precursor molecules is resistant to cleavage and probably represents denatured molecules.) In contrast, a matrix extract from *mas 1* mitochondria was at least 20-fold less active at 23°C and completely inactive at 0°C. A similar result was obtained with chemically pure, radioiodinated precursor to the F_1-ATPase β-subunit (Figure 5B).

The inability of *mas 1* matrix fractions to process these precursor polypeptides does not reflect any gross change of matrix composition: both type of mitochondria yield the same amount of soluble matrix proteins per mg of total mitochondrial protein (not shown) and both matrix fractions contain closely similar amounts of citrate synthase per mg matrix proteins (Figure 5D). Finally, the two matrix fractions contain virtually the same spectrum of polypeptide bands as detected by SDS-polyacrylamide gel electrophoresis (Figure 5C). Only two bands are greatly reduced (or missing) in the *mas 1* extract: a faint band of ~50 kd (arrow) and a prominent band at ~22 kd (asterisk). Further work is necessary to determine whether either of these two bands is related to the chelator-sensitive matrix protease. However, it is clear that the dramatic decrease in processing activity of *mas 1* mitochondria is not paralleled by a gross abnormality of their matrix space.

Discussion

We have shown that a yeast mutant temperature-sensitive for mitochondrial assembly has greatly lowered activity levels of a matrix protease involved in the maturation of imported mitochondrial precursor polypeptides. Mitochondria isolated from the mutant catalyze oxidative phosphorylation at high efficiency, exhibit respiratory control and import mitochondrial precursor polypeptides nearly as rapidly as mitochondria from the wild-type parent strain. However, they remove the transient pre-sequences from imported precursors only very slowly. Also, a soluble matrix fraction isolated from *mas 1* mitochondria contains at least 20-fold lower activity levels of the chelator-sensitive matrix protease than a matrix fraction from wild-type mitochondria. Thus, analysis of isolated mitochondria indicates that the *mas 1* mutation impairs processing, rather than translocation, of mitochondrial precursor polypeptides. Whether the *mas 1* defect is in the structural gene for the protease or in some regulatory gene cannot be determined from our studies.

The ability to observe a *mas 1* phenotype even with subcellular fractions was crucial for a more detailed characterization of this mutation. However, the results obtained *in vitro* differ in two respects from those obtained previously with intact *mas 1* cells. First, the *mas 1* mutant was initially selected as a temperature-sensitive mutant, but isolated *mas 1* mitochondria are already defective at room temperature. This discrepancy does not appear to be a serious one since pulse-chase experiments had already indicated that, even at room temperature, processing of the F_1-ATPase β-subunit was several fold slower in the *mas 1* mutant cells than in the wild-type cells (Figure 4 of Yaffe and Schatz, 1984). The difference between mutant and wild-type cells was further accentuated at 37°C, but the *mas 1* mutation clearly affected precursor processing even at 23°C. Since temperature sensitivity of a mutation usually reflects the restricted alteration of a protein, it is not unreasonable to expect that such an altered protein would be more labile than its wild-type counterpart during subcellular fractionation. Second, pulse-chase experiments with *mas 1* cells had shown that the F_1-ATPase β-subunit precursor that accumulated at the non-permissive temperature had not yet passed through the energy-dependent step of import and was, thus, probably located outside the mitochondrial inner membrane (Yaffe and Schatz, 1984). This accumulated precursor was converted to the mature β-subunit at the non-permissive temperature even though the rate of this maturation was between one and two orders of magnitude slower than with wild-type cells. In contrast, isolated *mas 1* mitochondria accumulate unprocessed precursor at, or inside the inner membrane (e.g., Figure 3) and this accumulated precursor cannot be chased to the mature form (not shown). This discrepancy cannot be readily explained at present. As the pulse-chase experiments with intact cells were done after the cells had been exposed to 37°C for 30 min, it is possble that this extended temperature block caused massive accumulation of precursors inside the mitochondria which in turn indirectly slowed down the movement of precursors from the cytosol into the mitochondria. With isolated *mas 1* mitochondria, precursor that had accumulated in the matrix (or as some transport complex within the inner membrane) might quickly become incompetent for subsequent processing. This discrepancy deserves re-investigation since we have recently found that the *mas 1* phenotype is already fully expressed after exposing the cells to 37°C for periods as short as 5 min (unpublished).

The accumulation of unprocessed precursors by energized

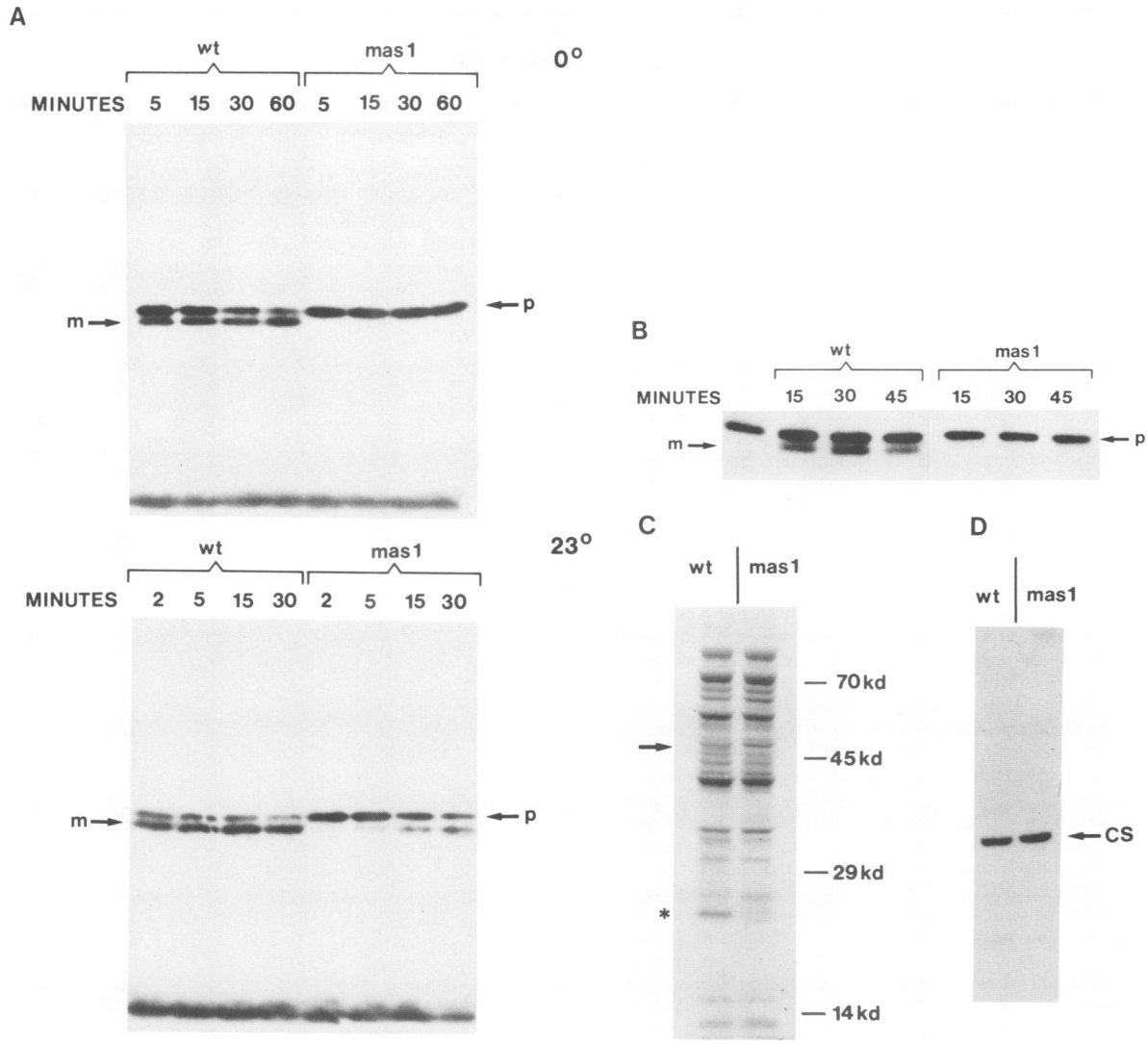

Fig. 5. The matrix fraction of *mas 1* mitochondria has greatly lowered levels of a protease activity that cleaves mitochondrial precursor polypeptides, but normal levels of other matrix proteins. Matrix fractions from wild-type (wt) mitochondria or *mas 1* mitochondria were prepared as described (Böhni *et al.*, 1983) but with the following modifications: (i) yeast cells were grown at 23°C in 12 l batches of semisynthetic medium containing 2% galactose, 0.1% glucose and harvested at an A_{600} = 4; (ii) cells and subcellular fractions were never exposed to temperatures above 23°C. (**A**) A protein containing the 53 amino-terminal residues of the cytochrome *c* oxidase subunit IV precursor fused to the amino terminus of mouse dihydrofolate reductase was synthesized *in vitro* in the presence of [^{35}S]methionine and 5 μl of the labeled mixture was incubated for the indicated times with 20 μl of matrix fraction (15 μg protein) at 23°C or at 0°C. Samples were mixed with 25 μl of 2 x concentrated SDS-sample buffer, heated to 95°C for 4 min, and analyzed by SDS-13% polyacrylamide gel electrophoresis and fluorography (see Figure 1). m and p, uncleaved and cleaved fusion protein, respectively. (**B**) Homogeneous, radioiodinated precursor to the yeast F_1-ATPase β-subunit (2.5 ng protein diluted as described in Materials and methods; ~2 x 10^9 c.p.m./mg) was incubated with 15 μg matrix fraction in a final volume of 25 μl at 23°C. Samples were analyzed as in **A** except that the dried gel was radioautographed. (**C**) Matrix proteins (50 μg) from either wild-type (wt) or *mas 1* mitochondria were electrophoretically resolved on an SDS-12% polyacrylamide gel and stained with Coomassie Brilliant Blue. The asterisk and the arrow identify a major and a minor protein band, respectively, which are present in the wild-type matrix fraction but absent in the *mas 1* matrix fraction. The cells had been grown at 23°C. (**D**) Matrix proteins (45 μg) from either wild-type (wt) or *mas 1* mitochondria were electrophoretically resolved on an SDS-10% polyacrylamide gel and subjected to immune blotting with rabbit antiserum against yeast citrate synthase (CS). The cells had been grown at 23°C.

mas 1 mitochondria supports the contention that import of radiolabeled precursors into mitochondria is not dependent upon removal of the transient pre-sequences (Reid *et al.*, 1982; Zwizinski and Neupert, 1983; Hurt *et al.*, 1985). However, processing appears to be essential for sustained growth since the cells stop growing at the non-permissive temperature after 2–3 generations. It is not clear whether this is caused by the accumulation of unprocessed precursors or the depletion of mature polypeptides within the mitochondria. In addition, it is possible that the *mas 1* mutation blocks functions other than proteolytic processing of precursors. The processing defect found with isolated *mas 1* mitochondria explains one of the most characteristic features of the *mas 1* phenotype: its pleiotropy. This feature is evident both with intact cells and with isolated mitochondria.

We have recently isolated fragments of yeast genomic DNA that restore temperature-resistant growth to *mas 1* mutant cells (R. Jensen, unpublished). Analysis of the cloned gene will allow us to determine if *MAS 1* is the structural gene of the matrix protease and allow further biochemical and genetic studies. By isolating and characterizing a large number of *mas* mutants one should thus be able to track down and characterize most of the components involved in mitochondrial protein import.

Materials and methods

Yeast strains, cell growth and subcellular fractionation

The parent strain AH 216 (*a leu 2 his 3 pho C pho E*) and the *mas 1* mutant derived from it (Yaffe and Schatz, 1984) were grown at 23°C on semisynthetic medium in the presence of 2% galactose to an $A_{600} = 3-4$. Mitochondria were isolated as described (Daum *et al.*, 1982) and assayed within 1–2 h. The precursor to the F_1-ATPase β-subunit was isolated from a *rho*$^-$ derivative of the yeast strain SF 747-19-d (α, *leu 2, his 4, ura 3, gal 2)* transformed with the yeast gene for the F_1-ATPase β-subunit on the yeast/*Escherichia coli* shuttle vector pFL-1 (G. Reid, unpublished).

Assays

Import and processing of ^{35}S-labeled precursor polypeptides was measured as outlined in Figure 1. Under these conditions, the production of processed precursors is roughly proportional to the amount of mitochondria added. The coupled transcription-translation system (Stueber *et al.*, 1984) was employed for the expression of cloned yeast genes as described (Hurt *et al.*, 1984a). Measurements of respiratory control and ADP/O ratios of isolated mitochondria are detailed in Figure 2. Published methods were used for measuring protein with bovine serum albumin as a standard (Lowry *et al.*, 1951), for SDS-polyacrylamide slab gel electrophoresis (Daum *et al.*, 1982), for fluorography (Hurt *et al.*, 1984a), for immune blotting (Haid and Suissa, 1983) and for quantifying the intensity of bands on X-ray film (Suissa, 1983).

Preparations

Homogeneous precursor to the yeast F_1-ATPase β-subunit was isolated essentially as described (Ohta and Schatz, 1984) except that precursor was isolated from a yeast transformant overproducing this precursor because it carried the corresponding gene on a multicopy plasmid (cf. above). The precursor polypeptide was radioiodinated in 3.4 M urea, 0.2 M potassium phosphate pH 7.5, 0.4% glucose with Enzymobeads (containing immobilized lactoperoxidase and glucose oxidase; BioRad Corp., USA) for 10 min at room temperature. The reaction was stopped by adding 2-mercaptoethanol to 0.9%, removing the Enzymobeads by centrifugation and passing the supernatant through a Sepahadex G-10 column equilibrated with 8 M urea, 1% 2-mercaptoethanol, 50 mM potassium phosphate pH 7.5 and 10 mg bovine serum albumin/ml. Fractions containing the radioiodinated precursor were frozen in small aliquots at −70°C. Immediately before use, an aliquot was thawed, diluted 100-fold with 10% glycerol, 0.1 M KCl, 1 mM $MgCl_2$, 20 mM Hepes-KOH pH 7.5 and then added to the assay mixture described in Figure 5B. While this renaturation protocol was probably not optimal, it was rapid and convenient; up to two-thirds of the labeled precursor molecules were renatured sufficiently to be processed by the solubilized matrix protease. Mitochondrial matrix fractions (Böhni *et al.*, 1983) and antiserum against yeast citrate synthase (Riezman *et al.*, 1982) were prepared as described. The genes coding for amino-terminal regions of the cytochrome *c* oxidase subunit IV precursor fused to the amino terminus of mouse dihydrofolate reductase are described in Hurt *et al.* (1984a, 1984b, 1985). Zymolyase was purchasd from Kirin Brewery Ltd., Japan and [^{35}S]methionine (> 1000 Ci/mmol) and carrier-free ^{125}-I from Amersham International, UK.

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References

Böhni,P., Gasser,S., Leaver,C. and Schatz,G. (1980) in Kroon,A.M. and Saccone,C. (eds.), *The Organization and Expression of the Mitochondrial Genome*, Elsevier/North Holland, Amsterdam, pp. 423-433.
Böhni,P., Daum,G. and Schatz,G. (1983) *J. Biol. Chem.*, **258**, 4937-4943.
Cerletti,N., Böhni,P. and Suda,K. (1983) *J. Biol. Chem.*, **258**, 4944-4949.
Daum,G., Böhni,P. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13028-13033.
Haid,A. and Suissa,M. (1983) *Methods Enzymol.*, **96**, 192-205.
Hay,R., Böhni,P. and Gasser,S. (1984) *Biochim. Biophys. Acta*, **779**, 65-87.
Horwich,A.L., Kalousek,F., Mellman,I. and Rosenberg,L.E. (1985) *EMBO J.*, **4**, 1129-1135.
Hurt,E.C., Pesold-Hurt,B. and Schatz,G. (1984a) *EMBO J.*, **3**, 3149-3156.
Hurt,E.C., Pesold-Hurt,B. and Schatz,G. (1984b) *FEBS Lett.*, **178**, 306-310.
Hurt,E.C., Pesold-Hurt,B., Suda,K., Oppliger,W. and Schatz,G. (1985) *EMBO J.*, **4**, 2061-2068.
Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J. Biol. Chem.*, **193**, 265-275.
McAda,P.C. and Douglas,M. (1982) *J. Biol. Chem.*, **257**, 3177-3182.
Miura,S., Mori,M., Amaya,Y. and Tatibana,M. (1982) *Eur. J. Biochem.*, **122**, 641-647.
Ohta,S. and Schatz,G. (1984) *EMBO J.*, **3**, 651-657.
Reid,G.A., Yonetani,T. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13068-13074.
Riezman,H., Hay,R., Witte,C., Nelson,N. and Schatz,G. (1983) *EMBO J.*, **2**, 1113-1118.
Schatz,G. and Butow,R.A. (1983) *Cell*, **32**, 316-318.
Stueber,D., Ibrahimi,I., Cutler,D., Dobberstein,B. and Bujard,H. (1984) *EMBO J.*, **3**, 3143-3148.
Suissa,M. (1983) *Anal Biochem.*, **133**, 511-514.
Yaffe,M.P. and Schatz,G. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4819-4823.
Zwizinski,C. and Neupert,W. (1983) *J. Biol. Chem.*, **258**, 13340-13346.
Zwizinski,C., Schleyer,M. and Neupert,W. (1984) *J. Biol. Chem.*, **259**, 7850-7856.

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