Precursor Binding to Yeast Mitochondria

A GENERAL ROLE FOR THE OUTER MEMBRANE PROTEIN Mas70p*

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Binding of precursors to import receptors on the mitochondrial surface is one of the earliest steps of protein import into mitochondria. In yeast, one of these receptors is a 70-kDa outer membrane protein termed Mas70p. Pulse-chase studies with intact yeast cells had indicated that Mas70p accelerates the import of all mitochondrial precursors tested. In contrast, import experiments with isolated mitochondria suggested that Mas70p accelerated import of only a subset of precursors (Hines, V., Brandl, A., Griffiths, G., Horstmann, H., Brutsch, H., and Schatz, G. (1990) EMBO J. 9, 3191–3200). To resolve this discrepancy, we have now studied the interaction of Mas70p-deficient and wild-type yeast mitochondria with a precursor (pre-alcohol dehydrogenase III) whose import into isolated mitochondria is not accelerated by Mas70p under the usual assay conditions. Mas70p enhanced binding of pre-alcohol dehydrogenase III to the surface of mitochondria in which the electrochemical potential across the inner membrane had been dissipated by an uncoupler; the bound precursor could be efficiently chased into the mitochondria if the potential was restored. The precursor to cytochrome c1 was also bound to mitochondria in a Mas70p-dependent manner. Mas70p also enhanced the direct import of pre-alcohol dehydrogenase III into isolated mitochondria, provided the precursor was first denatured with urea. Under these conditions, the import rate in vitro was more similar to that in intact cells. Mas70p had no effect on the binding or the import of artificial precursors containing mouse dihydrofolate as the "mature" domain. We conclude that Mas70p is an import receptor for most, if not all, authentic mitochondrial precursor proteins, but that its function is not always rate-limiting in import experiments with isolated mitochondria.

Several proteins of the mitochondrial outer membrane facilitate import of precursor proteins from the cytoplasm into mitochondria by binding precursors and delivering them to the import channels across the mitochondrial membranes (1–3). These import receptors can bind precursor proteins in the absence of an electrochemical potential across the inner membrane; when the potential is restored, the bound precursors are imported into the mitochondria. Studies with Saccharomyces cerevisiae and Neurospora crassa have identified several different import receptors (4–7). None of them appear to be essential for import, perhaps because the different receptors have overlapping functions (1). One of these receptors, termed Mas70p in S. cerevisiae and MOMP72 in N. crassa, is an outer membrane protein of approximate molecular mass 70,000. Mas70p contains a carboxyl-terminal 60-kDa domain which faces the cytoplasm, and a hydrophobic amino-terminal region which targets and anchors the protein to the outer membrane (8). Deletion of the MAS70 gene is not lethal to yeast cells, but retards growth on certain minimal media at high temperature (9).

The results obtained with yeast and N. crassa all suggest that Mas70p is not essential for mitochondrial protein import, but only accelerates this process. However, it has remained unclear whether Mas70p/MOMP72 is a general import receptor, or whether its receptor function is more restricted. Pulse-chase studies with wild-type and Mas70p-deficient yeast cells implied a general role, as Mas70p-deficient yeast cells imported all mitochondrial precursors tested several-fold more slowly than the corresponding wild-type cells (6). In contrast, import experiments with mitochondria isolated from these same yeast strains indicated that Mas70p acted only upon a subset of precursors (6): it accelerated import of the F1-ATPase β-subunit and of the ADP/ATP translocator, but not that of cytochrome b3, the mitochondrial isozyme III of alcohol dehydrogenase (ADHIII),1 or COXIV-DHFR (a fusion protein composed of the presequence of cytochrome oxidase subunit IV attached to mouse dihydrofolate reductase). An even greater precursor specificity was suggested by experiments with isolated N. crassa mitochondria: MOMP72 appeared to accelerate import of only the ADP/ATP translocator (5).

As mitochondrial protein import is much faster in intact cells than in the usual import assays with isolated mitochondria, we suspected that the divergent conclusions mentioned above might result from different rate-limiting steps in these two systems. Since Mas70p is not essential for protein import, it should accelerate this process only if its interaction with the precursor is rate-limiting. If another import step is rate-limiting in vivo, but not in vitro, the effect of Mas70p could only be detected in experiments with intact cells, but not with

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1 The abbreviations used are: pADHIII, precursor to the mitochondrial isozyme of yeast alcohol dehydrogenase; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DHFR, mouse dihydrofolate reductase; pADHIII-DHFR, a fusion protein containing the 27-amino acid presequence of pADHIII fused to the amino terminus of DHFR; pCOXIV-DHFR, a fusion protein containing the presequence of cytochrome oxidase subunit IV fused to the amino terminus of DHFR; kb, kilobase(s); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MEES, 4-morpholineethanesulfonic acid.
isolated mitochondria. The present study has attempted to test this hypothesis by exploiting two different in vitro assays: binding of precursors to untreated mitochondria, and import of urea-denatured precursors.

Protein transport across the mitochondrial inner membrane is blocked by collapsing the electrochemical potential across that membrane. Under these conditions, mitochondrial precursors bind to the outer membrane, but progress no further along the import pathway until the potential is re-established (10). This binding step represents a "partial" reaction of protein import; however, it does not involve turnover of receptor-precursor complexes, and should thus have different rate-limiting steps than complete import. Similarly, import of a precursor into isolated mitochondria is dramatically accelerated by denaturing the precursor, presumably because a low, rate-limiting step early in the import pathway is bypassed. Under these conditions, mitochondrial protein import in vitro becomes nearly as fast as import in vivo (11).

These two in vitro assays revealed that Mas70p facilitates the interaction of yeast mitochondria with all authentic precursors tested. Thus, Mas70p appears to be an import receptor for most, if not all authentic mitochondrial precursor proteins.

**MATERIALS AND METHODS**

*S. cerevisiae* Strains—The original mas70 disruption mutant had been generated by inserting the URA3 marker into the MAS70 gene at codon 106 of the open reading frame (9). In principle, the resulting disruption mutant might still synthesize an amino-terminal fragment of Mas70p; to exclude this possibility, we replaced the entire 5'-coding region of the MAS70 gene by the LEU2 gene by the following procedure: the 5'-region of the MAS70 gene (from 700 bp upstream of the initiating ATG up to codon 556 of the coding region) was subcloned from YEp13-70K (9) as a BamHI/EcoRV fragment into pBluescript KS (Stratagene). The 5'-end of the MAS70 gene (from -98 to +877 (codon 292)) was excised by BglII digestion, and the LEU2 gene was inserted in front of the truncated MAS70 gene as a 3.0-kb BglII fragment. This plasmid was digested with BamHI and KspI to generate a fragment for integrative transformation of strain YKX101. Cleavage of the linear fragment was stimulated by addition of phenylmethylsulfonyl fluoride to 1 mM. Samples were reisolated by centrifugation and analyzed by SDS-PAGE and fluorography. A photograph of the fluorogram is shown. This procedure was repeated with strain JKR101, a leu2-3,112 mutant, to exclude this possibility, we replaced the entire 5'-coding region of the MAS70 gene by the LEU2 gene by the following procedure: the 5'-region of the MAS70 gene (from 700 bp upstream of the initiating ATG up to codon 556 of the coding region) was subcloned from YEp13-70K (9) as a BamHI/EcoRV fragment into pBluescript KS (Stratagene). The 5'-end of the MAS70 gene (from -98 to +877 (codon 292)) was excised by BglII digestion, and the LEU2 gene was inserted in front of the truncated MAS70 gene as a 3.0-kb BglII fragment. This plasmid was digested with BamHI and KspI to generate a fragment for integrative transformation of strain JKR101 (MATa; ATCC 25657).

**Cell Growth and Isolation of Mitochondria**—Cells were grown to early stationary phase in semisynthetic medium containing 2% sorbitol and 0.6 M potassium phosphate, pH 7.4. Mitochondria were routinely isolated from this newly constructed Mas70p-deficient mutant, or from its wild-type parent, JKR101. For the experiments presented in Fig. 1, mitochondria were isolated from the S. cerevisiae strain D273-10B (MATa; ATCC 25657).

**For Assaying Binding of Precursors to the Mitochondrial Surface**—Mitochondrial isolation was necessary to reduce nonspecific binding to contaminating nonmitochondrial membranes. The mitochondrial suspension isolated by differential centrifugation was layered onto a discontinuous Nycodenz gradient (5 ml of 19% Nycodenz, 5 ml of 13% Nycodenz in 0.6 M sorbitol, 20 mM MES/KOH, pH 6.0) and centrifuged at 200,000 × g for 30 min (SW-41 rotor, Kontron ultracentrifuge). The purified mitochondria were removed from the 13/19% interface, diluted with 0.6 M sorbitol, 20 mM Hepes/KOH, pH 7.4, and collected by centrifugation at 12,000 × g for 10 min (SS-34 rotor, Sorvall centrifuge). This procedure rapidly and efficiently removed nonmitochondrial matter without affecting the import capability of the mitochondria. These purified mitochondria were used for all import and binding experiments.

**Import and Binding Assays**—The standard import buffer (Buffer A) contained 0.6 M sorbitol, 20 mM Hepes/KOH, pH 7.4, 2.5 mM unlabeled L-methionine, 40 mM KCl, 5 mM MgCl2, 1 mM EDTA, 2 mM K3P, 1 mM dithiothreitol, and 2 mg/ml bovine serum albumin.

![FIG. 1. pADHIII binds productively to mitochondria in the absence of a membrane potential. A, generation of the bound intermediate. Mitochondria (100 µg) were incubated for 10 min at 25 °C in a final volume of 400 µl in the presence of ATP and CCCP with 18 µl of reticulocyte lysate containing radioabeled pADHIII as described under "Materials and Methods." The sample was then divided in half, and mitochondria from each aliquot were reisolated through a sucrose cushion. For measuring the amount of bound precursor, the mitochondria of one aliquot were washed with 400 µl of Buffer A containing 0.1 µg/ml valinomycin and resuspended in 200 µl of valinomycin-containing Buffer A. For measuring the chase of bound precursor into the mitochondrion, mitochondria of the other aliquot were washed with 400 µl of Buffer A with 20 mg/ml bovine serum albumin (to remove CCCP from the organelles), resuspended in 200 µl of the same buffer with ATP mixture, and incubated for 5 min at 25 °C. For treatment with protease K (PROT.K), the samples were incubated for 15 min at 0 °C with 50 µg/ml proteinase K. Incubation was stopped by addition of phenylmethylsulfonyl fluoride to 1 mM. Samples were reisolated by centrifugation and analyzed by SDS-PAGE and fluorography. A photograph of the fluorogram is shown. P, precursor form; M, mature form. B, chase of the bound intermediate into mitochondria is less sensitive to low temperature than direct import of unbound precursor. Mitochondria (150 µg) were incubated for 5 min at 25 °C in Buffer A containing 5 µM CCCP and 1 mM ATP, but lacking dithiothreitol. For binding (BIND AND CHASE, upper panel), 25 µl of reticulocyte lysate containing pADHIII was added, followed by incubation for an additional 10 min at 25 °C. The mitochondria were then reisolated through a sucrose cushion and washed as described in A. To chase the bound pADHIII, the sample was divided into three equal aliquots; each aliquot was re-energized with ATP, mixed with 8 µl of reticulocyte lysate containing radioabeled pADHIII, and incubated at the indicated temperatures. Chase or import were stopped after 5 min by addition of valinomycin to 0.1 µg/ml, and samples were analyzed by SDS-PAGE and fluorography. C, generation of the bound intermediate is stimulated by ATP. Radioabeled pADHIII was bound to mitochondria as described in A, except that mitochondria and reticulocyte lysate were both preincubated with either 10 units/ml apharase (−ATP) or with ATP mixture (+ATP) for 10 min at 25 °C. During the subsequent binding incubation in the presence of CCCP, the assay mixture contained either apyrase (10 units/ml) (−ATP) or ATP mixture (+ATP). The mitochondria were reisolated and washed and one half of each sample was re-energized with ATP to chase the bound precursor as described in A. Binding and chase of prebound precursor were assessed as described under "Materials and Methods." For import (or binding) in the presence of ATP, an ATP mixture (generating final concentrations of 60 µg/ml creatine kinase, 4.5 mM creatine phosphate, and 1 mM ATP in the import assay) was added to the reaction. Unless stated otherwise, each import assay mixture contained 25 µg of mitochondrial protein in a final volume of 0.1 ml. The radioabeled precursors were purified pC0XIV-DHFR (13) or precursors synthesized in a nuclease-treated reticulocyte lysate by transcription/translation (14, 15) from plasmids encoding pre-cyto-
chrome c (16), pADHIII (17), and pADHIII-DHFR (18). After precursor synthesis, all lysates were centrifuged for 20 min at 150,000 × g in a Beckman Airfuge to remove ribosomes. Ten μl of lysate containing radiolabeled precursor was added to 90 μl of the import assay mixture to initiate binding or import. For each precursor, conditions were determined so that import was linear with time and with the concentration of mitochondria. Import was stopped by adding valinomycin to 0.1 μg/ml and apyrase to 10 units/ml. The mitochondria were reisolated by centrifugation and resuspended in SDS-PAGE sample buffer.

For binding and import experiments with urea-denatured PADHIII, reticulocyte lysate containing the radiolabeled precursor was depleted of ATP with 50 units/ml apyrase and adjusted to 66% saturation of ammonium sulfate. After 30 min at 0 °C, precipitated proteins (which included most of pADHIII) were collected by centrifugation, dissolved in 8 M urea, 10 mM Tris-HCl, pH 7.4, and diluted 20-fold into the mitochondrial suspension in import buffer. The final concentration of urea in the assay (0.4 M) does not inhibit import (11) or binding (not shown).

To assay for productive binding of precursors, the mitochondrial membrane potential was disrupted with the protonophore CCCP. Fifty μg of mitochondrial protein was incubated in 200 μl of Buffer A without dithiothreitol containing 6 μM CCCP for 5 min at 25 °C. For binding in the presence of ATP, ATP mixture (see above) was added during the incubation. For binding in the absence of ATP, mitochondria and reticulocyte lysate were pretreated with 10 units/ml and 50 units/ml apyrase, respectively, for 5 min at 25 °C. Precursor binding was initiated by adding 6–10 μl of reticulocyte lysate containing the radiolabeled precursor to the pretreated mitochondria. After 10 min at 25 °C, the mitochondria were resolated by centrifugation through a 25% sucrose cushion (400 μl) containing 20 mM Hepes/KOH, pH 7.4. For binding measurements, the mitochondria were resuspended and washed once with 400 μl of Buffer A without dithiothreitol containing 0.1 μg/ml valinomycin, resolated by centrifugation, and resuspended in SDS-PAGE sample buffer. Chasing the bound precursor into the mitochondria, the mitochondria were resuspended in 400 μl of Buffer A containing 20 mg/ml bovine serum albumin (serum albumin tightly binds CCCP and reverses its uncoupling effect on mitochondria), and resolated by centrifugation. To re-establish the membrane potential during the chase, washed mitochondria were resuspended in 200 μl of Buffer A containing 20 mg/ml bovine serum albumin, ATP mixure, 10 mM succinate, and 10 mM malate, and aerated as a thin layer in a flat-bottomed plastic vial for 5–10 min at 25 °C. Following the chase, the mitochondria were resolated and resuspended in SDS-PAGE sample buffer as above.

To quantify the amount of radiolabeled protein, samples were analyzed by SDS-PAGE, fluorography of the dried gels, and densitometry of the fluorograms (14). In all experiments, a standard lane was included that contained 10–20% of the total precursor which had been added to the import or binding assay. This standard lane was used to quantify the amount of bound or importable precursor as a percentage of the total precursor added to the assay.

RESULTS

The Precursor to Mitochondrial Alcohol Dehydrogenase Binds Productively to Uncoupled Yeast Mitochondria—The precursor to mitochondrial alcohol dehydrogenase (pADHIII) is one of three isozymes of alcohol dehydrogenase in S. cerevisiae; it is synthesized with a typical matrix-targeting sequence, imported into the mitochondrial matrix, and cleaved to its mature size by the matrix-processing protease (17, 18). Our previous work suggested that import of pADHIII into isolated yeast mitochondria is not accelerated by Mas70p (6). As discussed above, however, import into isolated mitochondria may have different rate-limiting steps than import in vivo; we therefore decided to test whether Mas70p promotes binding of the precursor to the surface of uncoupled mitochondria.

Mitochondria were pretreated with the protonophore CCCP to dissipate the electrochemical potential across the inner membrane and then incubated with radiolabeled pADHIII in the presence of ATP. After a 10-min incubation, more than 80% of the added precursor had bound to the mitochondria. The bound precursor retained its precursor, and was completely digested by added proteinase K, indicating that it had not been transported across the outer membrane (Fig. 1A, CHASE, −, PROT.K (+)).

This bound precursor could be chased into the mitochondria by re-establishing the potential across the inner membrane. As a result of this chase, virtually all of the bound precursor was cleaved to mature ADHIII, and about 50% of the cleaved molecules became inaccessible to added protease (Fig. 1A, CHASE (+)). Thus, at least 50% of the precursor molecules had bound "productively" to the uncoupled mitochondria. This percentage is probably an underestimation, as the cleaved, but protease-accessible molecules most likely represented partly translocated molecules spanning both membranes (19, 20).

In order to prove that the bound pADHIII was a genuine import intermediate, we had to exclude that its chase into the mitochondria reflected dissociation from the mitochondria followed by import from the unbound state. This possibility was rendered unlikely by the observation that the chase of bound precursor was less sensitive to low temperature than import of unbound precursor. The chase of bound pADHIII was equally efficient at 8, 15, and 25 °C; 49, 45, and 49% of added precursor was processed, respectively (Fig. 1B, BIND). In contrast, import of unbound precursor was inhibited at low temperature; at 8, 15, and 25 °C, 17, 33, and 58% of the added precursor was processed (Fig. 1B, DIRECT IMPORT). Most of the bound pADHIII is thus bound productively: it is a genuine import intermediate which has already passed the cold-sensitive import step.

Productive Binding of pADHIII to mitochondria was stimulated 2–4-fold by ATP (Fig. 1C; see also Fig. 2 below). Several other authentic mitochondrial precursors and DHFR-containing fusion proteins showed similar productive binding in this assay, although the stimulation by ATP appeared to vary between different precursors (see below).

Productive Binding of pADHIII Is Enhanced by Mas70p—To assess the role of Mas70p in the productive binding of pADHIII to the mitochondrial surface, we compared the binding capacity of mitochondria isolated from a wild-type yeast with those isolated from a Mas70p-deficient mutant. Alternative approaches to study the effect of Mas70p on binding would be to use antibodies directed against Mas70p, or to degrade Mas70p by a mild-protease treatment of intact mitochondria (6). However, our earlier studies had shown that a comparison of wild-type and Mas70p-deficient mitochondria is the most reliable method for assessing the role of Mas70p in protein import. If analyzed side-by-side immediately upon isolation, wild-type and Mas70p-deficient mitochondria exhibit virtually identical respiratory control ratios, and import several precursors proteins, including pADHIII, with equal efficiencies (6).

Productive binding of pADHIII to wild-type mitochondria was about 2-fold higher than to Mas70p-deficient mitochondria, regardless of whether it was measured in the presence or absence of ATP (Fig. 2A). The same difference between the two types of mitochondria was also found for total binding of pADHIII. When pADHIII was first denatured by urea and then presented to mitochondria, binding was no longer stimulated by ATP, but was still enhanced by the presence of Mas70p (Fig. 2B). Similar results were obtained when the binding capacity of wild-type mitochondria was measured before and after Mas70p had been removed by mild trypsin treatment (not shown).

Urea Denaturation of pADHIII Unmasks the Accelerating Effect of Mas70p on Import into Isolated Mitochondria—The results presented so far pose a dilemma: Mas70p enhances
Mitochondrial Import Receptor Mas70p

Fig. 2. Mas70p enhances binding of native or urea-denatured pADHIII to mitochondria. A, freshly isolated mitochondria from the wild-type (WT) or the Mas70p-deficient mutant (mas70) were tested for their ability to bind radiolabeled pADHIII. Binding reactions were done either in the absence (−ATP) or the presence (+ATP) of ATP. After binding, the mitochondria were reisolated and washed. One-half of the sample was analyzed directly for precursor binding, the other half was re-energized with ATP and incubated for an additional 5 min at 25°C to chase prebound precursor. See Fig. 1 for details. The open bar represents the amount of bound precursor, and the solid bar represents the amount of processed protein after chase. All values are expressed as percentage of the total amount of precursor added to the reaction. Binding and chase reactions with mitochondria from the wild-type or the Mas70p-deficient mutant were carried out as in A with the modifications described for urea-denatured precursor under “Materials and Methods.” The incubation time for the binding reaction was reduced to 3 min to minimize binding of refolded precursor. Other conditions were as described in A.

Productive binding of pADHIII to mitochondria (Fig. 2) yet has no effect on import of this precursor into isolated mitochondria (6). However, the role of Mas70p during import into isolated mitochondria might have been masked if the rate of import had been limited by the generation of an import-competent conformation of the precursor (11, 13) or by the release of precursor from a chaperone in the reticulocyte lysate (21). Either of these steps should be bypassed if the radiolabeled precursor is added to mitochondria in a urea-denatured state. Indeed, with some precursors, denaturation can accelerate import up to 70-fold (11).

To increase its import rate in vitro, radiolabeled pADHIII was denatured by urea before being presented to mitochondria. Denaturation accelerated import into wild-type mitochondria 3-5-fold. Import of un-denatured pADHIII into mitochondria increased linearly with time for up to 10 min at 15°C, whereas import of urea-denatured pADHIII was linear with time for only 2 min at this temperature (not shown). The upper panel of Fig. 3 compares the import kinetics of urea-denatured pADHIII into wild-type and Mas70p-deficient mitochondria. After 90 s, 23% of added denatured pADHIII was processed by wild-type mitochondria (Fig. 3, upper panel, pADHIII, WT). A significant fraction of pADHIII remained unprocessed, because import of urea-denatured precursors is often accompanied by increased nonproductive binding. The import kinetics of urea-denatured pADHIII into Mas70p-deficient mitochondria was significantly different: only 8% of the added precursor was processed after 90 s. Thus, import into Mas70p-deficient mitochondria was about 3-fold slower than import into wild-type mitochondria. Denaturation of the precursor had unmasked an accelerating effect of Mas70p on import into isolated mitochondria.

This conclusion was validated by two controls. First, import of un-denatured pADHIII into wild-type and Mas70p-deficient mitochondria occurred at identical rates (not shown, but see Ref. 6). Second, wild-type and Mas70p-deficient mitochondria imported urea-denatured COXIV-DHFR with comparable rates and efficiencies (Fig. 3, pCOXIV-DHFR). Thus, the different import rates shown in the upper panel of Fig. 3 were not caused by a selective effect of low urea concentrations on Mas70p-deficient mitochondria. The result shown in the lower panel of Fig. 3 also extends our earlier observation that import of artificial precursors containing DHFR as the “mature” domain is Mas70p-independent (see also below).

Mas70p Promotes Productive Binding and Import of Pre-cytochrome c1 by Isolated Mitochondria—Earlier pulse-chase experiments with intact cells had demonstrated that import of pre-cytochrome c1 into mitochondria is accelerated by Mas70p 3-4-fold (6). Fig. 4A shows that it also enhanced productive binding of this precursor to isolated mitochondria, both in the absence and presence of ATP. When the mitochondrial membrane potential was restored, 40-50% of the bound precursor was chased to process “intermediate” form (Fig. 4A, compare BIND (W) and CHASE (W)). Under these chase conditions, complete processing of pre-cytochrome c1 was not observed, since the second processing step requires addition of NADH (22). Productive binding of pre-cytochrome c1 to Mas70p-deficient mitochondria was 3-fold lower in the absence of ATP, and 2-fold lower in the presence of ATP, than binding to wild-type mitochondria.

Mas70p also accelerated the import of pre-cytochrome c1 into isolated mitochondria: import of this precursor into Mas70p-deficient mitochondria was 2.5-fold slower than into wild-type mitochondria (Fig. 4B). Unlike with pADHIII, this accelerating effect could be observed with the denatured precursor. In this experiment, import was assessed by conversion of the precursor to the intermediate form. As this conversion takes place in the matrix (23), it showed that the precursor’s amino-terminal domain has been transported across the inner membrane. Additional control experiments (not shown) verified that a large fraction of the resulting intermediate-sized molecules had also been transported across
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Discussion

Binding Assay—In this study we have asked whether the outer membrane protein Mas70p is an import receptor for only a few mitochondrial precursor proteins, or whether its function is more general. We suspected that the conflicting results obtained previously with intact yeast cells and isolated mitochondria might be reconciled if, for some precursors, Mas70p was a rate-limiting import catalyst in vivo but not during the usual import experiments with isolated mitochondria. We therefore assayed the effect of Mas70p on the productive binding of precursor proteins to de-energized mitochondria. Since this assay measures the end point, rather than the rate, of a partial reaction of import, uncertainties stemming from different rate-limiting steps should be minimized.

The bound precursor molecules can be efficiently chased into the mitochondria in a reaction that is faster, and less sensitive to low temperature, than import from the unbound

the outer membrane to the intermembrane space (see also Ref. 24).

Effect of Mas70p on Precursor Binding to Mitochondria Is Not Solely Determined by the Precursor’s Presequence—The results reported above showed that both pADHIII and precytochrome c bind to Mas70p early in the import pathway. In order to test whether this binding occurred via the precursors’ presequence, we fused the presequence of pADHIII to the amino terminus of DHFR and tested binding of this artificial precursor to mitochondria isolated from wild-type or Mas70p-deficient yeast. Fig. 5 shows that this fusion protein bound to uncoupled mitochondria and that 30% of the bound precursor molecules could be chased to a processed form when the mitochondria were re-energized. However, the binding of this fusion protein differed in two important respects from that of the authentic precursor which had served as the source of the presequence. First, only 20% of the added fusion protein was bound to the mitochondria. Second, productive binding to wild-type and to Mas70p-deficient mitochondria was identical. In addition, the two types of mitochondria imported this fusion protein equally fast, and to the same extent (data not shown). Thus, while this fusion protein bound productively to mitochondria, binding was inefficient, and not enhanced by Mas70p. This suggests that the presequence of pADHIII is not the sole determinant for productive binding to Mas70p.
state. These features are consistent with those of a true import intermediate, and validate the binding assay. 

Productive binding to the mitochondrial surface is stimulated by ATP, but the magnitude of this effect appears to vary between different precursors. With pADHIII, this effect is abolished when the precursor is presented to mitochondria in a denatured state. ATP might mediate the release of the precursor from an antifolding protein in the reticulocyte lysate, or could be necessary to induce a conformation of the precursor which is bound by Mas70p. The role of ATP in this binding reaction is being investigated further.

\textbf{Mas70p Is a General Import Receptor—}In vitro, Mas70p enhances productive binding of pADHIII to mitochondria, and accelerates import of denatured pADHIII into mitochondria, but does not accelerate import of undeveloped pADHIII. The usual import assays with isolated mitochondria may thus fail to detect the role of Mas70p in the import of precursor proteins, because import in vitro may have other rate-limiting steps than import in vivo. Mas70p also facilitates binding, and in vitro import, of pre-cytocrome c1. With this precursor, as with those reported earlier (6), its effect is seen even with the undeveloped precursor. The combined results from this study, and from our previous one (6), indicate that Mas70p is an import receptor for most, and perhaps all, authentic precursors.

\textbf{How Does Mas70p Recognize Mitochondrial Precursors?—}It is not clear whether Mas70p recognizes a precursor's presequence, a precursor's mature domain, or both. Mas70p does not appear to recognize a hybrid precursor whose presequence is derived from an authentic precursor (pADHIII) which does bind to Mas70p. These findings suggest that the precursor's presequence may not be the sole determinant for interaction with Mas70p. We cannot exclude that Mas70p also interacts with presequences, but we consider it likely that other outer membrane proteins are involved in presequence binding. Perhaps precursors bind sequentially or simultaneously to several outer membrane protein receptors. Mas70p might be involved in maintaining a precursor in an import-competent conformation by interacting with the mature domain.

Interaction of a precursor with Mas70p does not require that the precursor be bound to a cytosolic chaperone, as this interaction is still observed when a precursor is added to mitochondria by dialysis from 8 M urea. We consider it unlikely that a chaperone-precursor complex would reform under these conditions.

One possible interpretation of the results presented here is diagrammed in Fig. 6; precursors are normally presented to the mitochondrial import machinery as complexes with cytosolic antifolding proteins, such as chaperones of the 70-kDa heat shock protein family, which prevent aggregation or premature unfolding of the precursors. The precursors are released from the chaperone and bind to the mitochondrial outer membrane in a reaction requiring ATP hydrolysis by the chaperone. Productive binding to the mitochondrial surface is enhanced by Mas70p and perhaps by other import receptors, which together recognize the targeting information, maintain the precursor in an import-competent conformation, and deliver it to the import channels across the two mitochondrial membranes. Complete import and processing require ATP in the matrix and a membrane potential across the inner membrane. Transfer of some precursors to Mas70p may be rate-limiting during import experiments with isolated mitochondria, but not in intact yeast cells. Denaturation of these precursors bypasses ATP-dependent release from a chaperone which, for some proteins such as pADHIII, may be relatively slow in vitro. Denaturation of the precursor would accelerate delivery to Mas70p, making this receptor protein rate-limiting for import in vitro.

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