

A Chimeric Mitochondrial Precursor Protein With Internal Disulfide Bridges Blocks Import of Authentic Precursors into Mitochondria and Allows Quantitation of Import Sites

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Abstract. Bovine pancreatic trypsin inhibitor (which contains three intramolecular disulfide bridges) was chemically coupled to the COOH terminus of a purified artificial mitochondrial precursor protein. When the resulting chimeric precursor was presented to energized isolated yeast mitochondria, its trypsin inhibitor moiety prevented the protein from completely entering the organelle; the protein remained stuck across both mitochondrial membranes, with its

NH₂ terminus in the matrix and its trypsin inhibitor moiety still exposed on the mitochondrial surface. The incompletely imported protein appeared to "jam" mitochondrial protein import sites since it blocked import of three authentic mitochondrial precursor proteins; it did not collapse the potential across the mitochondrial inner membrane. Quantification of the inhibition indicated that each isolated mitochondrial particle contains between 10² and 10³ protein import sites.

To be translocated across a membrane, precursor proteins must at least temporarily assume a conformation which differs from that of the stably folded mature protein (Zimmermann and Meyer, 1986; Eilers and Schatz, 1988). For mitochondrial protein import, the necessity of such a conformational change was first shown (Eilers and Schatz, 1986) with an artificial precursor which contained a yeast mitochondrial presequence attached to mouse dihydrofolate reductase (DHFR)¹ (Hurt et al., 1984b). Binding of the DHFR inhibitor methotrexate fixed the tertiary structure of this precursor's DHFR moiety and blocked its import into mitochondria. A conformational change of this precursor appears to be rate-limiting for translocation since the rate of import into mitochondria is strongly enhanced by denaturing the precursor by urea (Eilers et al., 1988) or destabilizing its structure by point mutations (Vestweber and Schatz, 1988a). Ligand-induced inhibition of precursor translocation into mitochondria (Chen and Douglas, 1987) or chloroplasts (della Cioppa and Kishore, 1988) has recently been found by others as well. Also, posttranslational movement of an artificial precursor protein into the mammalian endoplasmic reticulum is blocked if the precursor contains an intramolecular disulfide bridge (Müller and Zimmermann, 1988). Finally, a large body of evidence indicates that trans-

location of a protein across its target membrane is correlated with a loosely folded structure (Wolfe and Wickner, 1984; Schleyer and Neupert, 1985; Randall and Hardy, 1986). The combined evidence strongly suggests that a tightly folded globular protein cannot traverse a biological membrane.

We hoped that a precursor protein carrying a tightly folded domain at its COOH terminus might be a useful tool to identify the site(s) at which proteins are transported from the cytoplasm into mitochondria. Such a precursor would correctly initiate import, but remain stuck in the import site, thereby jamming it. Schleyer and Neupert (1985) have previously described partly imported translocation intermediates spanning both mitochondrial membranes; however, since these intermediates were generated from trace amounts of radioactive precursors which had been synthesized *in vitro*, it could not be tested whether these intermediates had undergone a conformational change or whether they remained fixed in the import machinery itself. The small amounts of intermediates generated in this fashion also made it difficult to identify components of this import machinery.

In this study we prepared large amounts of an artificial precursor protein whose COOH-terminal domain contains three intramolecular disulfide bonds. This COOH-terminal domain interrupts the precursor's movement into mitochondria and causes it to jam the mitochondrial import sites. This allowed us, for the first time, to show that mitochondria contain a limited number (10²–10³) of import sites which are shared by several authentic precursor proteins during their import into the organelle.

1. *Abbreviations used in this paper:* BPTI, bovine pancreatic trypsin inhibitor; DHFR, dihydrofolate reductase; MBS, maleimidobenzoyl-*N*-hydroxysuccinimide ester; octyl-POE, octyl-polyoxyethylene; TC, tolylene-2,4-diisocyanate.

Materials and Methods

Synthesis of the Chimeric Precursor Protein

The chimeric precursor used in this study was constructed in five steps. In the first step, the first 22 residues of the yeast cytochrome oxidase subunit IV precursor were fused to the amino terminus of mouse DHFR by gene fusion technology (Hurt et al., 1984a). In the second step, the resulting cox IV-DHFR fusion protein was modified by site-directed mutagenesis of its gene so that its single cysteine residue represented the COOH-terminal amino acid (Vestweber and Schatz, 1988b). In the third step, the resulting DV12-cox IV-DHFR fusion protein was overexpressed in [³⁵S]O₄-labeled *Escherichia coli* and purified in microgram amounts. In the fourth step, bovine pancreatic trypsin inhibitor (BPTI) was activated at one of its five free amino groups with a maleimide group (cf., below). In the final step, the purified, radiolabeled DV12-cox IV-DHFR fusion protein was incubated with a 200-fold molar excess of the maleimide-derivatized BPTI for 30 min at room temperature, whereupon any unreacted maleimide groups were blocked by the addition of 2 mM dithiothreitol (DTT). The reaction product was directly used in import reactions. More than 90% of the DV12-cox IV-DHFR fusion protein was thereby converted to the BPTI-containing chimeric precursor.

Preparation of Maleimide-derivatized BPTI

BPTI (purchased as Aprotinin from Boehringer Mannheim GmbH, Mannheim, FRG) was dissolved to 5 mg/ml in 20 mM NaPi, pH 7.0. To 100 μ l of this solution, 1 mol equivalent (1 μ l of 25 mg/ml) of maleidobenzoyl-*N*-hydroxysuccinimide ester (MBS; Pierce Chemical Co., Rockford, IL) was added, and the mixture was allowed to react for 10 min at room temperature. Unreacted cross-linker was then removed by gel filtration on a 1-ml spin-column of Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) which had been equilibrated in a 1-ml syringe with 0.15 M NaCl, 10 mM NaPi, pH 7.0, and which was centrifuged for 5 min at 1,000 g. The flow-through (100 μ l) was mixed with 10 μ l of 1 M Tris-SO₄, pH 7.0, to block any residual unreacted cross-linker and the mixture was passed through another spin-column as described above. The flow-through from this second gel filtration was directly incubated with the DV12-cox IV-DHFR fusion protein as described above.

Conjugation of BSA to the Partially Imported Transmembraneous Chimeric Precursor

BSA (fatty acid-free; Sigma Chemical Co., St. Louis, MO) was conjugated to the bifunctional cross-linker tolylene-2,4-diisocyanate (TC) as described by Eytan and Schatz (1975). Isolated yeast mitochondria (containing 100 μ g protein) were allowed to import a mixture of the DV12-cox IV-DHFR fusion protein which had been COOH-terminally coupled to BPTI (cf., above) and the free fusion protein (190 ng protein total). After 30 min at 30°C, nonimported precursor molecules were digested by adding trypsin to 1.2 mg/ml and incubating the mixture for 25 min at 0°C. Trypsin was then inhibited by adding soybean trypsin inhibitor to 15 mg/ml and an additional incubation for 10 min at 0°C. The mitochondria were reisolated by centrifugation and suspended in 300 μ l of 20 mM KPi, pH 8.0, 0.6 M sorbitol containing 190 μ g–2.25 mg of BSA-TC, and incubated for 30 min at 30°C. Excess BSA-TC was blocked by adding 1 M Tris-SO₄, pH 8.0, to 0.3 M, 200 mM phenylmethylsulfonyl fluoride (in ethanol) to 0.5 mM, and incubating for 30 min at 30°C (keeping the sorbitol concentration at 0.6 M). As a control, mitochondria were carried through the same incubations either in the absence of BSA-TC, or with BSA-TC that had been pretreated with 0.3 M Tris-SO₄, pH 8.0, for 30 min at 30°C. Finally, mitochondria were reisolated by centrifugation and analyzed by SDS-12% PAGE and fluorography.

Import of Precursor Proteins into Isolated Mitochondria

Mitochondria were isolated (Daum et al., 1982) from the wild-type *Saccharomyces cerevisiae* strain D 273-10B (ATCC 25657; MAT α). Unless stated otherwise, import assays contained 200 μ g mitochondrial protein and 20 μ l (250 ng) of purified fusion protein or chimeric precursor in a final volume of 0.2 ml. Incubation was for 30 min at 30°C essentially as described (Gasser et al., 1982). For testing the inhibitory effect of the chimeric precursor on import of authentic precursors, 32 μ g of mitochondria were first incubated in a final volume of 0.24 ml for 18 min at 25°C with chimeric

precursor (125, 250, 375, or 500 ng) and then incubated for 10 min with 10 μ l of authentic precursors synthesized by transcription/translation (Hurt et al., 1984a). Import of the precursor to the F₁-ATPase β -subunit was tested at 25°C and that of the precursors to alcohol dehydrogenase III and to cytochrome oxidase subunit IV was tested at 15°C. As a control, the authentic precursors were imported into mitochondria which had been treated under identical conditions except that the chimeric precursor had been replaced by BPTI-MBS blocked by 2 mM DTT. To ascertain that pretreatment of mitochondria with the chimeric precursor did not cause collapse of the potential across the inner membrane, the potential was continuously recorded during the incubation with the chimeric precursor, using the potential-sensitive fluorescent indicator, (3,3')-dipropylthio-carbocyanine iodide (Sims et al., 1974) at 2.5 μ M. Excitation was at 620 nm and emission at 670 nm.

Controlled Disruption of Mitochondria with Octyl-Polyoxyethylene (octyl-POE)

Mitochondria (50 μ g) were incubated in a final volume of 100 μ l with 125 ng chimeric precursor for 25 min at 30°C in import buffer. They were reisolated by centrifugation (3 min at 15,600 g) and resuspended in 50 μ l of 0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, containing 0.2, 0.3, 0.4, or 0.5% (vol/vol) octyl-POE. After 10 min at 0°C, the samples were centrifuged in an Airfuge (Beckman Instruments Inc., Fullerton, CA) for 25 min at 30 psi (100,000 g) and supernatants and pellets were subjected to SDS-12% PAGE. The gels were analyzed either by direct fluorography, by staining with Coomassie Blue, or by immune blotting with antisera to citrate synthase (a marker for the matrix) or to porin (a marker for the outer membrane). Stained bands or bands on x-ray films were quantified by densitometric scanning using a CAMAG TLC scanner coupled to a CAMAG SP4290 integrator.

Miscellaneous

Published methods were used for SDS-12% PAGE and fluorography of dried gel slabs (Hurt et al., 1984b), preparation of antisera (Poyton and Schatz, 1975), immune blotting (Haid and Suissa, 1983), measurement of protein by the BCA procedure (Pierce Chemical Co.), and purification of the DV12-cox IV-DHFR fusion protein (Vestweber and Schatz, 1988b).

Results

The Chimeric Precursor Protein

The chimeric precursor protein used in this study was generated by exploiting recombinant DNA methods as well as chemical cross-linking of purified proteins. The construction started from a fusion protein which contained the first 22 residues of the yeast cytochrome oxidase subunit IV precursor fused to the NH₂ terminus of mouse DHFR. This fusion protein is imported, and cleaved, by mitochondria in vitro and in vivo (Hurt et al., 1984b, 1985). To cross-link BPTI specifically to the COOH terminus of this fusion protein, a cysteine residue was introduced at its COOH terminus and two internal cysteine residues were replaced with serine residues by site-directed mutagenesis (Vestweber and Schatz, 1988b). The mutated fusion protein was expressed and labeled with ³⁵S in *E. coli* and then purified.

The purified fusion protein was cross-linked at its COOH terminus to BPTI with the bifunctional cross-linker MBS. This reagent contains a maleimide group (which reacts with sulfhydryl groups) as well as an *N*-hydroxysuccinimide ester group (which reacts with free amino groups). The cross-linker was first added to an equimolar amount of BPTI; since BPTI lacks free sulfhydryl groups, but contains five free amino groups (the α -amino group at the NH₂ terminus and four lysine residues in positions 15, 26, 41, and 46), each BPTI molecule reacted on the average with the *N*-hydroxy-

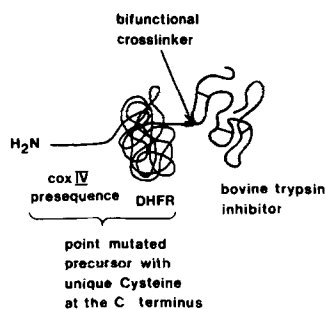


Figure 1. The chimeric precursor used in this study. *cox IV*, cytochrome oxidase subunit IV. The M_r of the presequence, the DHFR moiety, the chemical cross-linker, and the trypsin inhibitor moiety are 3, 21.6, 0.3, and 6 kD, respectively. The total M_r is 30.9 kD. See text for details. Note that only the DV12-*cox IV*-DHFR moiety of this protein is labeled with ³⁵S.

succinimide of one MBS molecule to yield a BPTI derivative with one covalently attached maleimide group. Excess MBS was removed by gel filtration and reaction with a >100-fold excess of Tris and the activated BPTI was reacted with the purified fusion protein. In most experiments, >90% of the fusion protein was converted to the BPTI-containing adduct. Since this adduct, like the mythological chimera, had been formed by the joining of three heterologous components, it is called a "chimeric precursor" in this study (Fig. 1).

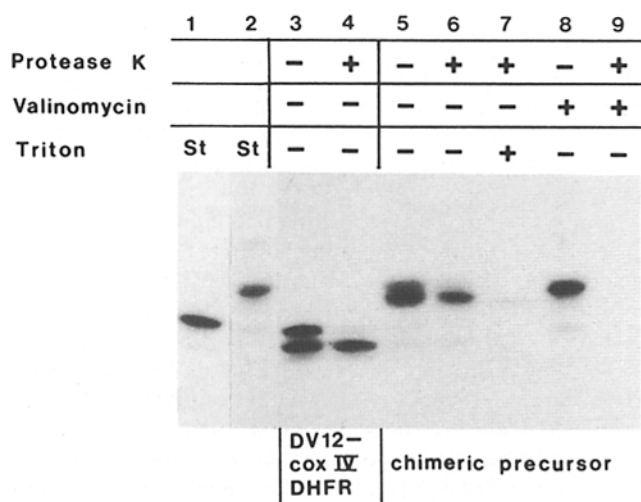


Figure 2. The chimeric precursor is processed, and its DHFR moiety is internalized, by isolated yeast mitochondria. The DV12-variant of the *cox IV*-DHFR fusion protein (250 ng, 1.7×10^5 dpm in 20 μ l) or an equimolar amount of the chimeric precursor (containing trypsin inhibitor attached to its COOH terminus) were incubated with yeast mitochondria (containing 200 μ g protein) for 30 min at 30°C in import buffer. The mitochondria were then reisolated and analyzed by SDS-12% PAGE and fluorography. A photograph of the fluorogram is shown. Lanes 1 and 2, 20% of the amount of purified fusion protein or of the chimeric precursor, respectively, that were added to each import assay. About 20–30% of the added proteins were imported as shown by cleavage of their presequence (lanes 3 and 5) and inaccessibility of their labeled DHFR moiety to externally added proteinase K (lanes 4 and 6). Lysis of the mitochondria with the detergent Triton X-100 abolished the protease protection of the DHFR moiety (lane 7). Cleavage of the presequence was dependent on import into the organelles since it was blocked by 10 μ g/ml of the uncoupler valinomycin (lanes 8 and 9).

The Chimeric Precursor Becomes Stuck Across the Mitochondrial Membranes

When the chimeric precursor was added to energized yeast mitochondria, its presequence was cleaved off, indicating that its NH₂ terminus had penetrated across both mitochondrial membranes into the matrix space (Fig. 2, lane 5). Its labeled DHFR moiety was also internalized since it was inaccessible to externally added proteinase K (Fig. 2, lane 6) unless the mitochondrial membranes were lysed by the detergent Triton X-100 (Fig. 2, lane 7). However, this experiment does not give information on the location of the precursor's BPTI moiety since this moiety is unlabeled and inherently resistant to proteinase K (not shown).

Controlled extraction of mitochondria with detergent provided the first indication that the imported chimeric precursor had not completely moved into the matrix space (Fig. 3). Mitochondria were first allowed to import either the BPTI-free fusion protein or the chimeric precursor and then exposed to increasing concentrations of the nonionic detergent octyl-POE. The mixtures were separated into supernatants and pellets by brief ultracentrifugation and each fraction was tested for the presence of submitochondrial marker proteins (Fig. 3, A and B) as well as for the presence of the imported labeled proteins (Fig. 3 C). The combined data of Fig. 3 show that exposure of mitochondria to 0.5% octyl-POE effected a clear-cut separation of matrix contents (markers, citrate synthase, and a characteristic 90-kD protein) from integral membrane proteins (markers, outer membrane porin, and the adenine nucleotide translocator). These extraction conditions solubilized essentially 100% of the imported, cleaved BPTI-free fusion protein (Fig. 3 C, top), but none of the cleaved chimeric protein (Fig. 3 C, bottom). Since the cleaved chimeric protein is inherently soluble in 0.5% octyl-POE (not shown), the data of Fig. 3 indicate that the cleaved chimeric precursor is still tightly associated with mitochondrial membranes.

To prove that the chimeric precursor's BPTI moiety had failed to enter the mitochondria, we tested its accessibility to an externally added, membrane-impermeable cross-linker. Such a cross-linker is BSA (68 kD) derivatized with isocyanate groups (BSA-TC; Eytan and Schatz, 1975). These groups spontaneously form a stable urea bridge with free amino groups; if a protein on the mitochondrial surface reacts with BSA-TC, its M_r will be increased by multiples of 68 kD (depending on how many cross-links it forms), resulting in a dramatic mobility shift upon analysis by SDS-PAGE.

In the experiment shown in Fig. 4, mitochondria were first allowed to import a mixture of the BPTI-free fusion protein and the chimeric protein; they were then reisolated by centrifugation and nonimported labeled proteins were degraded by externally added protease; the protease was inhibited and the mitochondria were reacted with increasing concentrations of BSA-TC; finally, the mitochondria were analyzed by SDS-PAGE and fluorography. The data showed that increasing concentrations of BSA-TC derivatized ~50% of the cleaved, imported chimeric molecules, but essentially failed to derivatize the cleaved, imported BPTI-free fusion protein.

We conclude that at least 50% of the imported chimeric molecules are stuck across the two mitochondrial membranes, with the DHFR moiety in the matrix and the BPTI moiety on the mitochondrial surface.

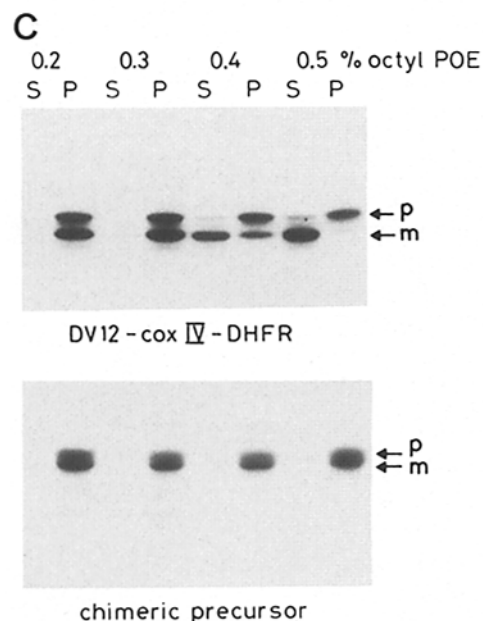
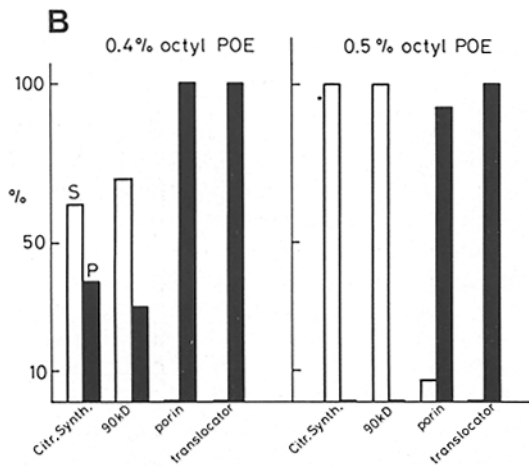
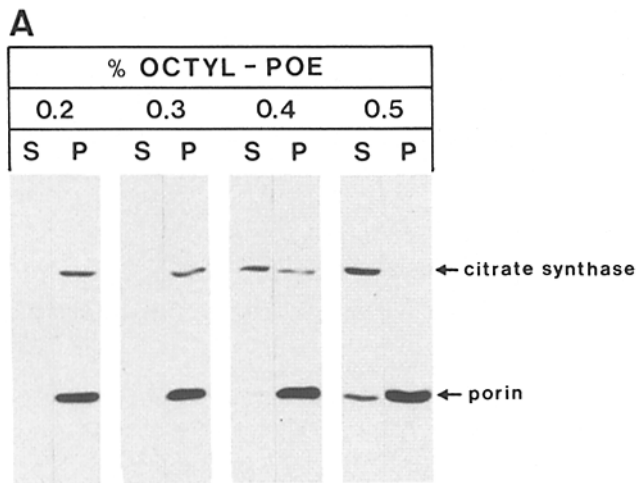


Figure 3. The partially imported chimeric protein remains associated with the mitochondrial membranes. The subfractionation procedures are as follows. (A) Immune blots. Mitochondria were lysed with the indicated concentrations of the nonionic detergent octyl-POE and separated into supernatant (S) and pellet (P) by ultracentrifugation (100,000 g for 25 min). Samples were analyzed by immune blotting with antisera against citrate synthase (a matrix

The data of Fig. 4, by themselves, do not show that the externally located part of the transmembrane precursor is, in fact, the BPTI moiety. However, this point is documented by the data of Fig. 2 which show that the entire DHFR moiety (which is labeled at many sites throughout its polypeptide chain) is inaccessible to externally added protease.

The Partially Imported Chimeric Precursor Blocks Import of Three Different Authentic Precursors

The experiments described so far suggest that the chimeric precursor correctly initiates translocation, but fails to complete it because its COOH-terminal BPTI moiety cannot be accommodated by the mitochondrial translocation machinery. If the partially translocated chimeric protein remains stuck within the translocation machinery, it should block import of authentic precursors into mitochondria.

This is indeed the case. When mitochondria were first allowed to import large amounts of the chimeric precursor (3.9–15.6 ng/mg mitochondrial protein), they lost the ability to import subsequently added precursors of cytochrome oxidase subunit IV, alcohol dehydrogenase isozyme III, or the F₁-ATPase β-subunit (Fig. 5). This inhibition was seen under conditions in which the rate of import was linearly dependent on the amount of mitochondria added (see Materials and Methods; Ohba and Schatz, 1987). No such inhibition was seen if, under identical conditions, the chimeric precursor was replaced by BPTI-MBS whose maleimide group had been blocked by DTT. To exclude the possibility that excess, nonimported chimeric precursor molecules simply bound or precipitated the authentic precursors, the authentic precursors were also added to mitochondria after these had been reisolated from the first incubation with the chimeric precursor; the observed inhibition was thereby not altered (not shown).

To test whether the partly imported chimeric protein affected the integrity of the mitochondria, we measured the potential across the inner membrane during the entire 18-min incubation with the highest concentration of chimeric precursor used in the competition experiments. At the end of the 18-min period, the potential had decreased by only 20%; the same small drop was seen if the mitochondria were incubated under the same conditions with the chimeric precursor omitted. In both cases, addition of valinomycin completely abolished the potential (not shown). This excludes the possibility that the inhibitory effect of the partly im-

marker) and porin (an integral protein of the outer membrane). (B) Quantification. The efficiency of the subfractionation was checked by densitometric quantification of the immune blots shown in A, of the Coomassie Blue-stained bands of a prominent 90-kD protein of the mitochondrial matrix, and of the adenine nucleotide translocator of the mitochondrial inner membrane. The amount of unextracted protein detected in the pellets of samples treated with 0.2% octyl-POE was taken as 100%. Open bars, supernatants; solid bars, pellets. (C) Extraction behavior of the partially imported chimeric precursor. The subfractionation procedure documented in A and B was applied to mitochondria which had been allowed to import either the DV12-variant of the cox IV-DHFR fusion protein or the chimeric precursor; supernatants (S) and pellets (P) were analyzed by SDS-12% PAGE and fluorography. Uncleaved (precursor, p) and cleaved (mature, m) forms of each protein.

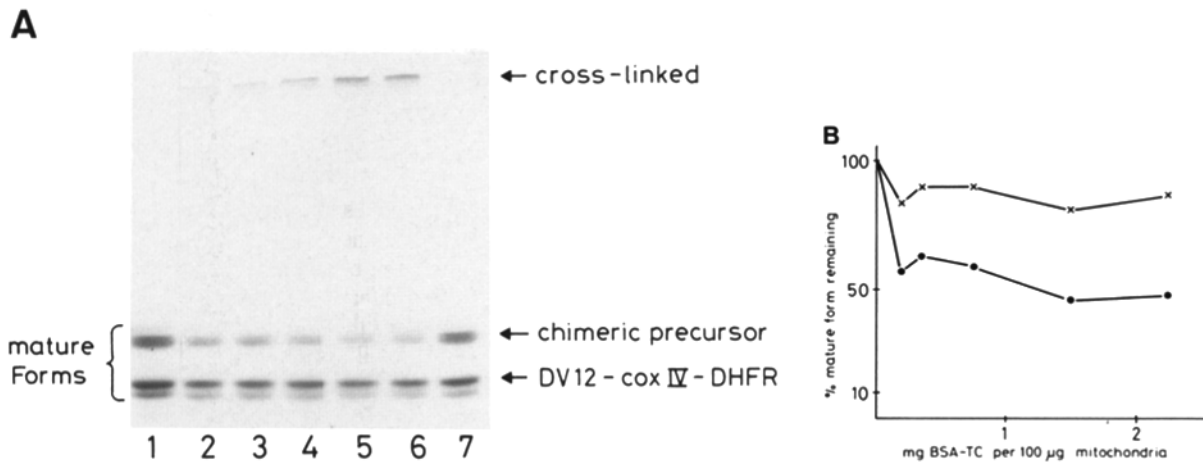


Figure 4. The partially imported chimeric precursor has been cleaved at its NH₂ terminus by the matrix protease, yet is still exposed on the mitochondrial surface. (A) The DV12-variant of the cox IV-DHFR fusion protein and the chimeric precursor were imported into isolated yeast mitochondria for 30 min at 30°C. Nonimported molecules were then degraded by added trypsin (1.2 mg/ml for 25 min at 0°C), trypsin was inhibited by excess soybean trypsin inhibitor (15 mg/ml), and the reisolated mitochondria (100 µg) were then reacted with BSA-TC in a final volume of 300 µl. After blocking excess BSA-TC, mitochondria were analyzed by SDS-12% PAGE and fluorography. As controls, mitochondria were either incubated without BSA-TC (lane 1) or "mock treated" with the highest amount (2.25 mg) of BSA-TC which had been blocked with excess Tris before being added to mitochondria (lane 7). Mitochondria were reacted with 190 µg (lane 2), 375 µg (lane 3), 750 µg (lane 4), 1.5 mg (lane 5), and 2.25 mg (lane 6) of BSA-TC. The mature forms of the labeled precursor proteins and molecules cross-linked to BSA-TC (*cross-linked*) are indicated on the sides. (B) Quantitation of the fluorogram shown in A. The fluorogram was scanned and the percentage of noncross-linked, imported fusion protein (×) or chimeric precursor (●) was plotted against the amount of BSA-TC that had been added to the mitochondria.

ported chimeric precursor merely reflects mitochondrial uncoupling.

We conclude that the partly imported chimeric protein represents a true import intermediate, that this intermediate remains associated with the import sites, and that this site is shared by several authentic mitochondrial precursor proteins.

Quantitation of Mitochondrial Import Sites

Since the chimeric precursor appears to be a specific, irreversible inhibitor of mitochondrial import, it can be used to titrate the mitochondrial import sites. Fig. 5 B shows that import of authentic precursors was almost completely blocked if 40 pmol of chimeric precursor had been partly imported by 1 mg of mitochondrial protein. This amounts to 2.4×10^{13} molecules of precursor/mg mitochondrial protein. Isolated yeast mitochondria can be approximated by spheres with a diameter of 0.8 µm. If one assumes that each of these spheres contains 10% (wt/vol) protein, then 1 mg of mitochondrial protein represents 3.7×10^{10} mitochondrial particles. Based on this approximate calculation, each fully inhibited isolated mitochondrial particle has imported on the order of 600 transmembraneous chimeric proteins.

Discussion

In this study we have described an artificial mitochondrial precursor protein that jams the mitochondrial import machinery. This feature must be caused by the COOH-terminal BPTI moiety since the BPTI-free fusion protein is completely imported into the soluble matrix space. Also, a very similar variant of this fusion protein does not block import

of authentic precursors at comparable concentrations (Endo and Schatz, 1988). It is likely that BPTI cannot move across a membrane because its three intramolecular disulfide bridges prevent it from assuming a loose, translocation-competent structure. We have not attempted to prove this rigorously since this was not the major aim of our work. In principle, the incomplete translocation of the chimeric precursor could also have resulted from the COOH-terminally attached cross-linker, or from the fact that linkage to BPTI via the epsilon amino group of any of the four internal lysine residues produces a branched polypeptide chain. We do not favor this possibility since replacement of the BPTI moiety by horse heart cytochrome c also produces branched precursors which are, however, completely imported into the matrix space (Vestweber and Schatz, 1988b). We conclude that a tightly folded globular protein the size of BPTI (~6 kD) cannot be transported across mitochondrial membranes.

The transmembrane location of the incompletely imported chimeric precursor resembles that of the translocation intermediates generated by Schleyer and Neupert (1985) with trace amounts of radiolabeled precursors by either low temperature or by attaching antibodies directed against the mature moiety. We, like they, conclude that the precursors are trapped at sites of close contact between the two mitochondrial membranes. As first suggested by Butow and his colleagues, these sites appear to be the entry points for cytoplasmically made proteins into mitochondria (Kellems et al., 1975; Ades and Butow, 1980; Suissa and Schatz, 1982; Schwaiger et al., 1987). Mitochondrial subfractionation has indeed shown that the transmembraneous chimeric protein is specifically associated with a membrane fraction exhibiting properties expected of "contact sites" (Pon, L., and T.

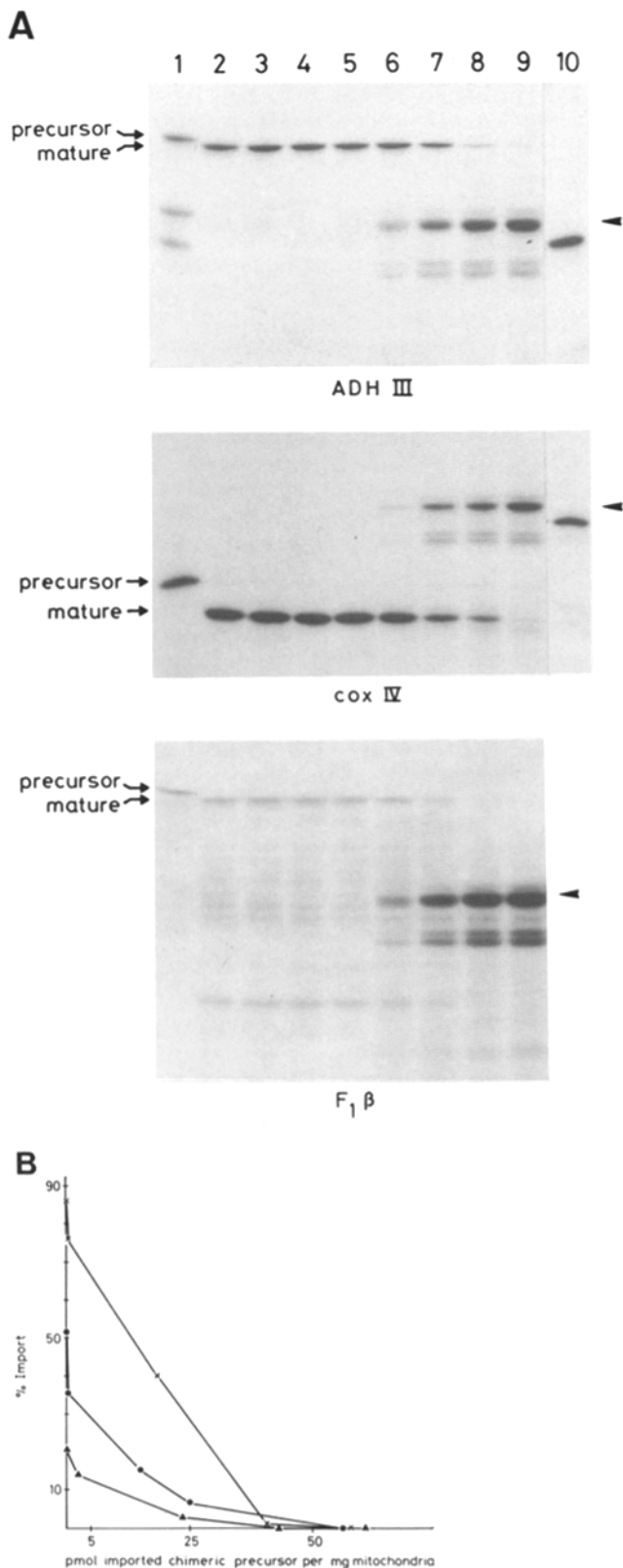


Figure 5. The partially imported, transmembrane chimeric precursor blocks import of authentic precursors. Mitochondria (32 μ g protein) were first incubated with the indicated amounts of the chimeric precursor for 18 min at 25°C in a final volume of 240 μ l. They were then allowed to import radiolabeled precursors of alcohol dehydrogenase isozyme III (*ADH III*), of cytochrome oxidase subunit IV (*cox IV*), and of the F_1 -ATPase β -subunit ($F_1\beta$). These

Moll, manuscript in preparation). Since $\sim 90\%$ of the processed chimeric precursor was found in this "contact site fraction," most of the processed precursor appears to remain stuck in the import site.

Import of the chimeric precursor (and, by implication, also that of the BPTI-free fusion protein) must share at least one step with the import of authentic precursors. This agrees with the previous findings that import of the BPTI-free fusion protein closely resembles import of authentic precursors in many different respects (Hurt et al., 1984a, b, 1985). The fusion protein is, thus, a valid model for authentic precursors except that its DHFR moiety appears to be more tightly folded (Endo et al., 1988).

Import of authentic precursors can also be blocked by chemically synthesized presequence peptides (Gillespie et al., 1985; Ono and Tuboi, 1988) or by mitochondrial precursor proteins lacking a cleavable presequence (Mori et al., 1985; Pfaller and Neupert, 1987). While these results suggest the existence of a finite number of mitochondrial import sites, they are difficult to interpret since the inhibitory peptides or precursors should engage the import machinery only transiently. In addition, mitochondrial prepeptides are amphiphilic and readily interact with lipid phases (Roise and Schatz, 1988) which could further lower the fraction of added peptides that is available to specific interaction with the import machinery.

In contrast, the chimeric protein blocked import of authentic precursors at very low concentrations ($<0.1 \mu\text{M}$) and after mitochondria had imported only ~ 40 pmol of precursor/mg mitochondrial protein. If we assume that the precursor is imported as a monomer, that all of it becomes stuck in a transmembrane orientation, and that the mitochondrial particles isolated by us contain 10% (wt/vol) protein, each mitochondrial particle should contain 10^2 – 10^3 import sites. Even though many of these assumptions may be questioned, the calculated number of import sites is in reasonable agreement with the observation that mitochondria isolated from bovine liver contain ~ 100 membrane contact sites per mitochondrial particle (Hackenbrock, 1968).

Since the chimeric precursor can be purified in appreciable amounts (60–75 μ g; Vestweber and Schatz, 1988b) it should be a valuable tool for identifying components of the mitochondrial import machinery. Such experiments are currently underway.

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authentic precursors had been synthesized by transcription/translation in vitro. (A) Fluorograms. Arrowheads, the position of the processed form of the chimeric precursor. Arrows, the positions of the precursor and mature forms of the authentic precursors. Lane 1, 20% of the authentic precursor added to each import assay; lanes 2–5, import of authentic precursor by four identical samples of control mitochondria; lanes 6–9, import of authentic precursor by mitochondria that had been preincubated with 125, 250, 375, and 500 ng, respectively, of chimeric precursor; lane 10, 35 ng of labeled purified fusion protein. (B) Quantitation of fluorograms. The fluorograms shown in A were scanned and the percentage of added authentic precursor which had been imported into mitochondria was plotted against the amount of processed chimeric precursor associated per mg mitochondrial protein. (▲) pre- $F_1\beta$; (●) pre-cox IV; (×) pre-ADH III.

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