Tight folding of a passenger protein can interfere with the targeting function of a mitochondrial presequence

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The first seven residues of the yeast cytochrome oxidase subunit IV presequence are insufficient to target attached mouse dihydrofolate reductase into isolated yeast mitochondria. However, the targeting function of this truncated presequence can be restored by presenting the fusion protein to isolated mitochondria either as nascent, unfolded chains, or as full-length chains whose dihydrofolate reductase moiety had been destabilized either by urea treatment or by point mutations. The targeting efficiency of a mitochondrial presequence can thus be strongly influenced by the conformation of the attached 'passenger protein'. These results also underscore the difficulty of defining a 'minimal' mitochondrial targeting signal.

Key words: mitochondrial import/presequences/protein conformation/protein translocation/targeting

Introduction

Mitochondria consist of several hundred proteins, yet only a few of these are encoded by the mitochondrial genome and synthesized within the organelle. All the rest are encoded by nuclear DNA, synthesized on cytoplasmic ribosomes and imported into the organelle (for review see Verner and Schatz, 1988). Mitochondrial proteins are generally synthesized in the cytosol as precursors which carry an N-terminal extension referred to as a presequence. Once imported into the mitochondrial matrix, the presequence is typically cleaved off by a chelator-sensitive protease, which generates the 'mature' form of the protein.

Much of what we know about the signals that target proteins to mitochondria has been elucidated by studying fusion precursor proteins consisting of the presequence of a mitochondrial protein fused to the N terminus of a 'passenger protein' (for review see Hurt and van Loon, 1986). The passenger protein can be a mitochondrial protein lacking its own presequence or a protein not normally imported into mitochondria. Such chimeric precursor proteins are often efficiently imported by mitochondria *in vitro* or *in vivo*. This has led to the view that all of the information necessary to correctly target a precursor protein to mitochondria is (in many cases) present in its N-terminal presequence.

However, presequences and their attached proteins have presumably evolved together, thereby providing the

opportunity for the development of specific interactions between these two domains. A selective advantage for such an interaction would be increased targeting efficiency of the precursor protein itself. Indeed, there is evidence to indicate that the 'mature' moiety of a mitochondrial precursor protein can affect the ability of its presequence to direct proper targeting and import. Van Steeg *et al.* (1986) have shown that the first 34 amino acids of the precursor to the mitochondrial matrix enzyme superoxide dismutase efficiently target attached mouse dihydrofolate reductase (DHFR) but not yeast invertase, into isolated yeast mitochondria. Similarly, Emr *et al.* (1986) showed that at least 169 N-terminal residues of the F₁-ATPase β -subunit precursor are required to direct mitochondrial import of *Escherichia coli* β -galactosidase, while no more than 39 residues are required if yeast invertase is the passenger protein. While these studies indicate that a passenger protein can affect the ability of a presequence to direct mitochondrial import, they do not identify the features of the passenger protein that are responsible.

In this study we show that the function of a mitochondrial presequence can be strongly influenced by the conformation of the passenger protein.

Results

Only nascent, but not completed, chains of a truncated fusion protein are imported into isolated yeast mitochondria

Hurt *et al.* (1985) have examined the mitochondrial import of fusion proteins consisting of the first 22, 16, 12, 9 and 7 residues of the mitochondrial cytochrome oxidase subunit IV presequence fused to the N terminus of mouse DHFR; only fusion proteins containing at least the first 12 residues of the mitochondrial presequence were imported into isolated yeast mitochondria. Neither the first nine nor seven residues of the subunit IV presequence were sufficient to direct import of DHFR into mitochondria.

To check whether this result depends on the conformation of the DHFR moiety, the fusion proteins containing the first 22 and seven residues of the subunit IV presequence fused to mouse DHFR [the COXIV(1–22)–DHFR and COXIV(1–7)–DHFR fusion proteins respectively] and authentic mouse DHFR were synthesized by *in vitro* transcription/translation in a nuclease-pretreated reticulocyte lysate. Nascent chains of the proteins were separated from the completely synthesized forms (Verner and Schatz, 1987) and separately added to isolated yeast mitochondria.

Figure 1 (top panel) confirms that completed as well as nascent chains of the fusion protein containing the 22-residue presequence are efficiently imported by energized mitochondria (Verner and Schatz, 1987); they were re-isolated with the mitochondria (lanes 2 and 8) and protected from externally added protease (lanes 3 and 9) unless the mitochondrial membranes were disrupted with detergent

Fig. 1. Isolated yeast mitochondria import only nascent, but not completed chains of the COXIV(1-7)-DHFR fusion protein. COXIV(1-22)-DHFR (top panel), COXIV(1-7)-DHFR (middle panel) and authentic mouse DHFR (lower panel) were synthesized by coupled transcription/translation in a nuclease-treated reticulocyte lysate for 8 min at 25°C in the presence of [³⁵S]methionine. Synthesis was stopped by the addition of an equal volume of buffer containing 200 µg/ml cycloheximide, 2 mM MgCl₂, 20 mM Hepes, pH 7.4. Post-ribosomal supernatant (containing the completely synthesized protein) was obtained by centrifuging the cycloheximide-arrested translation reaction mixture for 25 min at 130 000 g at 4°C. The ribosomal pellet was resuspended in 20 mM Hepes, pH 7.4, 1 mM MgCl₂. It was centrifuged a second time as above, resuspended in 20 mM Hepes, pH 7.4, 10 mM EDTA and incubated for 10 min at 0°C to dissociate ribosomes. Residual particles were removed by a third centrifugation as above; the final supernatant was used as a source of nascent chains. Post-ribosomal supernatants (10 µl) or isolated nascent chains (30 µl) were then incubated with 200 µg isolated yeast mitochondria for 20 min at 30°C (see Materials and methods). Where indicated, mitochondria were de-energized by 2 µg/ml valinomycin. The mitochondria were then reisolated by centrifugation and analyzed by 15% SDS-PAGE gels and fluorography. Where indicated, mitochondria were first treated with 250 µg/ml proteinase K (either in the presence or absence of 0.5% Triton X-100) for 15 min at 0°C and then with 1 mM PMSF before electrophoretic analysis. **Lane 1**, 20% of the labeled isolated nascent chains which were added to the mitochondrial samples shown in lanes 2-6; **lane 2**, after incubation with energized mitochondria; **lane 3**, energized mitochondria, treated with proteinase K; **lane 4**, after incubation with de-energized mitochondria; **lane 5**, de-energized mitochondria, treated with proteinase K; **lane 6**, after incubation with energized mitochondria, treated with proteinase K and Triton X-100; **lane 7**, 20% of the labeled post-ribosomal supernatants (completed chains) which were added to the mitochondrial samples shown in lanes 8 and 9; **lane 8**, after incubation with energized mitochondria; **lane 9**, energized mitochondria, treated with proteinase K.

(lane 6). No import was seen with mitochondria de-energized by valinomycin (shown in lanes 4 and 5 for nascent chains). The mol. wt distribution of the nascent chains bound to

de-energized mitochondria differed from that of chains imported into energized mitochondria. This difference may be caused by non-specific proteolytic degradation of chains bound to the mitochondrial surface (Verner and Schatz, 1987). Import of completed COXIV(1-22)-DHFR was accompanied by the removal of its presequence by the matrix located chelator-sensitive protease, resulting in a lower mol. wt 'mature' form (lanes 8 and 9).

Figure 1 (middle panel) also confirms that the first seven residues of the subunit IV presequence are inactive in transporting *in vitro* synthesized completed DHFR chains into mitochondria. The completely synthesized COXIV(1-7)-DHFR protein recovered in the post-ribosomal supernatant (lane 7) was neither bound (lane 8) nor imported (lane 9) by isolated yeast mitochondria. Unexpectedly, however, nascent chains of the protein were efficiently imported (lane 2); >20% of the amount added became resistant to externally added protease (lane 3) unless detergent was added to disrupt the mitochondrial membranes (lane 6). Import of COXIV(1-7)-DHFR nascent chains was potential dependent; no protease-protected chains were observed if the mitochondria were treated with valinomycin (lane 5). Thus, completely synthesized COXIV(1-7)-DHFR is not imported into isolated mitochondria, while its C-terminally truncated nascent chains are.

Since neither completely synthesized authentic mouse DHFR nor its nascent chains were imported by isolated yeast mitochondria (Figure 1, bottom panel), import of COXIV(1-7)-DHFR nascent chains requires the first seven amino acids of the cytochrome oxidase subunit IV presequence.

COXIV(1-7)-DHFR nascent chains are incompletely folded

Mouse DHFR has a compact globular structure (Volz *et al.*, 1982) which is highly protease resistant. This tightly folded DHFR domain is also present in the COXIV(1-22)-DHFR fusion protein (Eilers and Schatz, 1986; Endo and Schatz, 1988). In contrast, nascent chains of COXIV(1-22)-DHFR are much more protease sensitive and thus probably incompletely folded (Verner and Schatz, 1987). The same is true for COXIV(1-7)-DHFR (Figure 2). When a mixture of completed and nascent chains was incubated with increasing concentrations of proteinase K, the completed chains yielded the protease-resistant DHFR 'core' typical of a folded DHFR moiety (Eilers and Schatz, 1986; van Loon *et al.*, 1986). This 'core' is not clearly resolved from undigested COXIV(1-7)-DHFR in the gel system used in Figure 2, since this system was chosen for maximum visualization of nascent chains. In contrast to the completed protein, nascent chains were much more sensitive and were digested without the appearance of any stable intermediate. We conclude that the nascent chains of COXIV(1-7)-DHFR are less tightly folded than the completed chains.

Completed COXIV(1-7)-DHFR is imported into isolated mitochondria if denatured with urea

Completed COXIV(1-7)-DHFR might not be imported by isolated mitochondria because the truncated presequence does not sufficiently protrude from the folded protein to interact with the mitochondrial import machinery. Such 'masking' of the presequence should not occur with unfolded nascent chains. This hypothesis predicts that even the

Fig. 2. Nascent COXIV(1-7)-DHFR chains are more sensitive to digestion by proteinase K than completed chains. COXIV(1-7)-DHFR was synthesized by coupled transcription/translation in a nuclease-treated reticulocyte lysate for 8 min at 25°C in the presence of [35 S]methionine. Protein synthesis was stopped by the addition of an equal volume of buffer containing 200 μ g/ml cycloheximide, 2 mM MgCl_2 , 20 mM Hepes, pH 7.4. Aliquots were incubated for 20 min at 0°C with the indicated final incubations of proteinase K (μ g/ml) in 20 mM Hepes, pH 7.4, 100 mM KCl, 10 mM MgCl_2 , 1 mM dithiothreitol. Digestion was stopped by adding PMSF to 1 mM and the digestion products were analyzed on 15% SDS-PAGE gels and by fluorography.

Fig. 3. Urea-denatured completed COXIV(1-7)-DHFR is imported into isolated mitochondria. COXIV(1-7)-DHFR was synthesized as in Figure 1. Protein synthesis was stopped by 100 μ g/ml cycloheximide and the mixture was centrifuged for 25 min at 130 000 g at 4°C to sediment ribosomes with attached nascent chains. The post-ribosomal supernatant containing completely synthesized COXIV(1-7)-DHFR was removed and divided into two equal aliquots. One aliquot was made 6 M in urea and incubated on ice for 60 min, the other aliquot was incubated on ice for 60 min without the addition of urea. The urea-denatured COXIV(1-7)-DHFR (lower panel) and non-treated COXIV(1-7)-DHFR (upper panel) were incubated with 200 μ g isolated mitochondria for 20 min at 30°C (see Materials and methods). Where indicated, mitochondria were de-energized by 2 μ g/ml valinomycin. The mitochondria were then reisolated by centrifugation and analyzed on 15% SDS-PAGE gels and by fluorography. Where indicated, mitochondria were first treated with 250 μ g/ml proteinase K, in the presence or absence of 0.5% Triton X-100, for 15 min at 0°C and then with 1 mM PMSF before electrophoretic analysis. **Lane 1**, 20% of the labeled COXIV(1-7)-DHFR samples which were added to the mitochondria shown in lanes 2-6; **lane 2**, after incubation with energized mitochondria; **lane 3**, energized mitochondria, treated with proteinase K; **lane 4**, after incubation with de-energized mitochondria; **lane 5**, de-energized mitochondria, treated with proteinase K; **lane 6**, after incubation with energized mitochondria, treated with proteinase K and Triton X-100.

completed protein would be importable if it is first denatured with urea. This is indeed the case (Figure 3). When the completed, *in vitro* synthesized fusion protein was incubated in 6 M urea for 1 h on ice and then diluted into a suspension

Fig. 4. Protease sensitivity and *in vitro* import of COXIV(1-7)-DHFR destabilized by site-directed mutagenesis. Proteinase K digestion was performed on COXIV(1-7)-DHFR (top panel, left side) and COXIV(1-7)-DHFR mutated at positions 157 and 183 of DHFR (lower panel, left side) exactly as in Figure 2. Numbers 0-20 indicate the final proteinase K concentration in μ g/ml. Import into isolated yeast mitochondria of COXIV(1-7)-DHFR (upper panel, right side) and the mutated COXIV(1-7)-DHFR construct (lower panel, right side) was assayed as in Figure 1. **Lane A**, 10% of the labeled protein added to the mitochondrial samples shown in lanes B-E; **lane B**, after incubation with energized mitochondria; **lane C**, energized mitochondria, treated with proteinase K; **lane D**, after incubation with de-energized mitochondria; **lane E**, de-energized mitochondria, treated with proteinase K.

of isolated yeast mitochondria, it was bound by energized mitochondria (lower panel, lane 2) and a small, but significant fraction of the molecules was imported, becoming protected from externally added protease (lane 3) unless the mitochondrial membranes were disrupted by detergent (lane 6). Import of urea-denatured COXIV(1-7)-DHFR was potential dependent; no protease-protected radiolabeled protein was detectable if the mitochondria were uncoupled by valinomycin (lane 5). The import efficiency ($\sim 5\%$) of urea-denatured COXIV(1-7)-DHFR was lower than that of its nascent chains (Figure 1). This probably reflects the rapid refolding of DHFR-containing fusion proteins upon dilution of urea (Endo and Schatz, 1988).

Completed COXIV(1-7)-DHFR is imported into isolated mitochondria if the DHFR moiety is destabilized by point mutations

The introduction, by site-directed mutagenesis, of cysteine residues into the DHFR moiety of a COXIV-DHFR fusion protein destabilizes the protein (Vestweber and Schatz, 1988). We therefore constructed a fusion protein in which the truncated COXIV presequence was fused to a DHFR moiety modified by the introduction of cysteine residues at positions 157 and 183 of the DHFR sequence. Figure 4 illustrates both the protease sensitivity (left side of panels) and importability (right side of panels) of COXIV(1-7)-DHFR (upper panel) and of the COXIV(1-7)-DHFR variant modified by the introduction of two cysteines (lower panel). The modified DHFR moiety is more sensitive to

proteolytic degradation than the unmodified one, suggesting that it is less tightly folded. It is also efficiently imported by isolated mitochondria. In the experiment shown in Figure 4, >60% of the full-length chains added to mitochondria were imported. Import was potential dependent; it was abolished if the mitochondria were uncoupled by valinomycin (lane E).

Thus, folded COXIV(1–7)–DHFR is not imported into mitochondria whereas its loosely folded nascent chains, urea-denatured completed chains or the destabilized disulfide form are imported.

Discussion

In this study we describe an artificial precursor protein which is only imported into isolated mitochondria if the native structure of its DHFR moiety has been disrupted. This shows directly that the effectiveness of a presequence can depend on the conformation of the attached 'passenger' protein.

Our present data add a new dimension to the view that proteins can only move through membranes in a conformation that is different from their native one (Eilers and Schatz, 1988). The dependence of protein import on the conformation of a precursor protein may not only be related to the membranes' inability to translocate folded precursors but also to the membranes' inability to recognize and properly interact with the presequence. A loose conformation of a precursor in the cytoplasm may therefore assure a productive interaction with the target membrane. This notion is consistent with previous studies which suggested that the presequence of the full-length COXIV–DHFR fusion protein could not penetrate into natural or artificial membranes unless the DHFR moiety was partly unfolded (Endo and Schatz, 1988). Translocation of the precursor across the membrane may require yet additional energy-dependent conformational changes.

Interaction of a signal peptide with its passenger protein has been elegantly studied with the precursors to ribose- and maltose-binding proteins, two proteins exported from the cytoplasm to the periplasm in *E. coli* (Collier *et al.*, 1988; Liu *et al.*, 1988; Park *et al.*, 1988). With these authentic precursors, the signal peptide significantly retards refolding of the passenger part upon denaturation by chaotropes. Also, the export deficiency caused by a small deletion within the signal peptide of pre-maltose-binding protein can be partly suppressed by a mutation within the 'mature' part of the precursor. This intragenic suppressor mutation slows the rate of refolding of the mature moiety following denaturation, suggesting that it biases the 'race' between export and refolding in favor of the export process. As in the case of COXIV(1–7)–DHFR, the signal peptide of fully folded forms of secreted proteins may not be able to properly interact with the membrane.

Finally, our finding that only destabilized forms of COXIV(1–7)–DHFR are imported into mitochondria highlights the difficulty in defining a 'minimal' mitochondrial targeting sequence. Previous studies with the subunit IV presequence had suggested that at least 12 residues were required for mitochondrial targeting. However, we now show that the 'minimal sequence' may be even shorter and that the minimal length of the presequence appears to be inversely related to the stability of the DHFR moiety. A further complication is introduced by the fact that, *in vivo*,

many precursors may be prevented from folding in the cytosol by specific proteins which are only now being identified (for review see Verner and Schatz, 1988); the action of these proteins can probably not be reliably assessed in the commonly used heterologous protein-synthesizing systems.

Materials and methods

In vitro transcription/translation

Plasmids encoding either the first 22, 7 or 0 residues of the presequence of yeast cytochrome oxidase subunit IV fused to mouse DHFR (Hurt *et al.*, 1985) were transcribed *in vitro* (Stueber *et al.*, 1984) and transcripts were translated in a nuclease-treated reticulocyte lysate supplemented with hemin, creatine phosphate, creatine kinase, the 19 amino acids except methionine, 130 mM KCl, 2 mM MgCl₂ (Pelham and Jackson, 1976) as well as 250 µg/ml calf liver tRNA, 50 U human placental RNase inhibitor/ml and 1 mCi of [³⁵S]methionine (>1000 Ci/mmol) per ml (Hurt *et al.*, 1984). Translation was performed at 25°C, usually for 8 min, followed by addition of an equal volume of Arrest Buffer (200 µg/ml cycloheximide, 2 mM MgCl₂, 20 mM Hepes, pH 7.4). Nascent chains were separated from completed protein essentially as described (Verner and Schatz, 1987), except that no attempt was made to deplete ATP from the preparation of nascent chains.

A plasmid encoding the first seven residues of cytochrome oxidase subunit IV fused to DHFR containing cysteine at positions 157 and 183 of DHFR was constructed by exchanging restriction fragments between the plasmid encoding COXIV(1–7)–DHFR and a similar plasmid kindly contributed by Dr D. Vestweber, who had introduced the cysteines by site-directed mutagenesis as described (Vestweber and Schatz, 1988). Published methods were used for DNA manipulations (Maniatis *et al.*, 1982).

Protein import into isolated mitochondria

Yeast mitochondria were isolated (Daum *et al.*, 1982) from the wild-type *Saccharomyces cerevisiae* strain D273-10B (α; ATCC 25657). Mitochondria were incubated with radiolabeled proteins essentially as described by Gasser *et al.* (1982). The incubation (total volume 0.2 ml) contained 200 µg mitochondrial protein, either 30 µl isolated nascent chains or 10 µl of post-ribosomal supernatant derived from *in vitro* translation, 40 mM KCl and, where indicated, 2 µg/ml valinomycin. After 20 min at 30°C, the mitochondria were reisolated by centrifugation through a sucrose cushion. For digestion of externally located polypeptides following the import reaction, the mitochondria were incubated for 15 min at 0°C with 250 µg/ml proteinase K. The protease was inhibited with 1 mM PMSF and the mitochondria were reisolated as above.

Miscellaneous

Published procedures were used to analyze labeled translation products by SDS–PAGE and fluorography (Hurt *et al.*, 1984). Protein was measured by the BCA procedure as described in a company brochure ('Previews') of Pierce Chemical Co.

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