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Import of Proteins into Mitochondria

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Import of proteins into mitochondria

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A mounting body of evidence suggests that cytoplasmically synthesized proteins destined to be imported into the mitochondrial interior must at least partly unfold to penetrate across the mitochondrial membranes. During post-translational import, this unfolding process appears to be a major rate-limiting step. It can be blocked by ligands that stabilize the protein's native conformation and appears to be accompanied by the cleavage of ATP outside the mitochondrial inner membrane.

The problem

Much is known about the signals that guide cytoplasmically synthesized proteins to their specific intramitochondrial location (Hurt & van Loon 1986; Schatz 1987). However, very little is known about how these proteins can penetrate across the mitochondrial membranes. This process may in part be aided by the amphiphilic nature of mitochondrial presequences (Roise *et al.* 1986), but also appears to involve a proteinaceous 'import machinery': protein import into isolated mitochondria can be blocked by treating the mitochondrial surface with various proteases (Zwizinski *et al.* 1983; Riezman *et al.* 1983) or with Fab fragments directed against a group of 45 kDa outer membrane proteins (Ohba & Schatz 1987).

The identity and, indeed, the function or functions of the mitochondrial 'import machinery' are still unknown. Here we summarize recent evidence that suggests that one of the functions of this machinery may be to unfold a protein before its passage across the mitochondrial membranes. Although this model is still tentative, it makes specific predictions that can be experimentally tested.

The experimental system

Most of the experiments to be discussed here were carried out with an artificial precursor protein that consists of the presequence of subunit IV of yeast cytochrome oxidase fused to all 187 residues of mouse dihydrofolate reductase (DHFR, a cytosolic enzyme). If this fusion protein is synthesized *in vitro* and tested with isolated mitochondria (Hurt *et al.* 1984) or expressed in yeast cells and tested for its subcellular location (Hurt *et al.* 1985) it exhibits all the properties of a typical mitochondrial precursor protein. It is rapidly and efficiently imported into mitochondria; import is dependent on an energized inner membrane; and import is followed by the cleavage of the attached presequence by a chelator-sensitive processing protease in the mitochondrial matrix.

We have now expressed this protein in *E. coli* in the presence of [^{35}S]methionine and purified 100–200 μg of it by conventional procedures (Eilers *et al.* 1986). The final product is radiochemically and chemically nearly pure; it exhibits DHFR activity, suggesting that its DHFR moiety is properly folded; and if added to energized yeast mitochondria in the absence of other cell fractions, it is imported and cleaved to 'mature' DHFR. This highly purified,

enzymically active and import-competent precursor protein has offered unique possibilities for testing the importance of protein folding in protein transport across the mitochondrial membranes.

Import of the fusion protein is blocked by ligands binding tightly to DHFR

Import to the subunit IV–DHFR fusion protein into yeast mitochondria is inhibited by methotrexate and similar substrate analogues (Eilers *et al.* 1986). Detailed X-ray crystallographic studies have shown that these analogues bind tightly to the active site of DHFR and stabilize its folded structure (Volz *et al.* 1982). The analogues do not block import of precursors lacking a DHFR moiety; however, as expected, they do protect the DHFR moiety of the subunit IV–DHFR fusion protein against attack by several proteases. The inhibition of import by methotrexate suggested that the DHFR moiety of the fusion protein must at least partly unfold to enter mitochondria. How extensive this unfolding must be remains open, however.

Independent evidence from Randall & Hardy (1986) showed that the ability of maltose-binding protein to be exported from the *E. coli* cytoplasm correlated with a protease-sensitive (and presumably loosely folded) state of that protein.

Protein import into mitochondria requires both ATP and an energized inner membrane

DHFR is a tightly folded, globular, monomeric protein whose unfolding is almost certainly endergonic. If it is indeed unfolded during import into mitochondria, how is this process fuelled?

It was recently discovered that import of proteins into mitochondria requires not only an energized inner membrane (Schleyer *et al.* 1982; Gasser *et al.* 1982) but also ATP (Pfanner & Neupert 1986; Eilers *et al.* 1987). Our evidence on this point is summarized in figure 1. We also found that ATP can be replaced by GTP, but not by CTP, TTP or non-hydrolyzable ATP analogues. ATP appears to exert its effect outside the inner membrane: added ATP can support protein import even if translocation of ATP across the mitochondrial inner membrane is blocked by carboxyatractyloside.

Import of the incompletely folded fusion protein is very rapid and requires little or no added ATP

We speculated that ATP might in part be required to provide energy for the unfolding of the fusion protein. In that case, loosely or incompletely folded conformers of the protein might require little or no ATP for import. We tested this idea by two different sets of experiments.

In the first approach, we prevented correct folding of the subunit IV–DHFR fusion protein by synthesizing the protein in a reticulocyte lysate and interrupting synthesis with cycloheximide while most of the chains were still incomplete and bound to tRNA molecules (Verner & Schatz 1987). As these peptidyl tRNA molecules remained attached to ribosomes,

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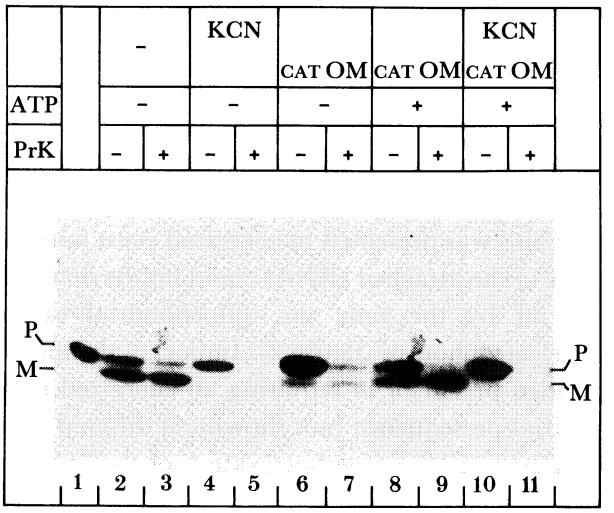

FIGURE 1. Import of a purified precursor protein requires both a membrane potential and ATP. Purified labelled precursor protein (1×10^5 counts per minute (cpm); 3×10^8 cpm mg^{-1} protein) was incubated with isolated yeast mitochondria in the presence of succinate and L-malate and, where indicated, with KCN, oligomycin (OM), or carboxyatractyloside (CAT). Mitochondria were either left untreated or treated with proteinase K (PrK) after import to digest precursor bound to the surface. They were then reisolated by centrifugation and analysed by SDS-polyacrylamide gel electrophoresis and fluorography. Lane 1 contains 10% of the radiolabelled fusion protein, which was added to each mitochondrial sample. (From Eilers *et al.* (1987).)

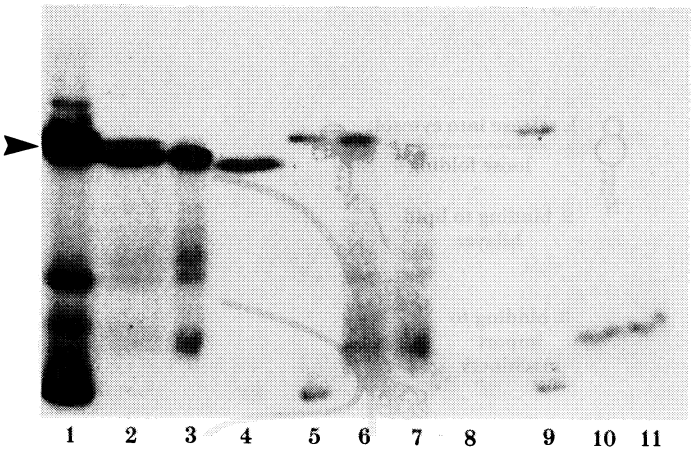

FIGURE 2. Import of nascent precursor chains into isolated mitochondria requires an energized inner membrane, but little, if any, added ATP. Radiolabelled nascent chains of the subunit IV–DHFR fusion protein were mixed with radiolabelled completed fusion protein that had been purified from *E. coli*. The mixture was assayed for import into isolated yeast mitochondria that had been pretreated with 23 µg ml^{-1} efrapeptin to block mitochondrial ATPase. Where indicated, ATP (2 mM), glycerol kinase (2 units ml^{-1}) and glycerol (12 mM) or valinomycin (2 µg ml^{-1}) (to uncouple mitochondria) were also added. Two samples were incubated without mitochondria to check for aggregation of the labelled precursor chains. All samples (total volume 200 µl) were incubated in air as shallow layers in plastic tubes for 20 min at 25 °C and centrifuged for 10 min at 15000 rev. min^{-1} in an Eppendorf centrifuge; the pellets were analysed by SDS–15% polyacrylamide gel electrophoresis followed by fluorography. Where indicated, mitochondria were treated with proteinase K (with or without 0.5% Triton X-100) followed by phenylmethylsulphonyl fluoride before electrophoretic analysis. Lanes: 1, 20% of the mixture of completed and incomplete precursor chains that was added to the samples shown in lanes 2–11; 2, after incubation with respiring mitochondria, plus ATP; 3, respiring mitochondria, ATP, proteinase K; 4, respiring mitochondria, ATP, proteinase K, Triton; 5, as 2, but no mitochondria added; 6, respiring mitochondria plus glycerol kinase; 7, respiring mitochondria, glycerol kinase, proteinase K; 8, respiring mitochondria, glycerol kinase, proteinase K, Triton; 9, as 6, but no mitochondria added; 10, uncoupled mitochondria, ATP, proteinase K; 11, uncoupled mitochondria, glycerol kinase, proteinase K. Arrow indicates purified precursor protein. (From Verner & Schatz (1987).)

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they could be partly purified by sedimenting the ribosomes, washing them by resuspension and recentrifugation, dissociating the ribosomes with EDTA, and removing ribosomal subunits by a final centrifugation. The released peptidyl tRNA molecules varied in length and appeared to be incompletely folded because they were much more sensitive to proteinase K than the completed precursor protein. It is most likely that correct folding was prevented by the attached tRNA, even with those chains that had been nearly completed. When this mixture of radiolabelled nascent chains was presented to energized yeast mitochondria, the chains were rapidly imported. The fate of the attached tRNAs could not be determined with certainty, but they had been cleaved off by the time the mitochondrial samples were analysed by SDS–polyacrylamide gel electrophoresis (figure 2). Import of the incomplete chains resembled that of the completed, folded precursor in requiring an energized inner membrane (figure 2, lane 10) and a mitochondrial presequence (not shown); it differed from that of the completed precursor protein in being insensitive to methotrexate (not shown) and requiring little, if any added ATP (compare lanes 3 and 7, figure 2).

In the second approach, radiolabelled subunit IV–DHFR fusion protein was purified from *E. coli*, denatured by 8 M urea and quickly diluted to a final urea concentration of 0.6 M. This procedure yielded a protein that was incorrectly folded as judged by the following four criteria: (i) it lacked DHFR activity; (ii) it was highly sensitive to trypsin; (iii) its trypsin-sensitivity was not decreased by the substrate analogue methotrexate; (iv) its presequence was no longer cleaved off by the purified matrix protease. (This protease fails to act on denatured precursor proteins; cf. Boehni *et al.* (1983).)

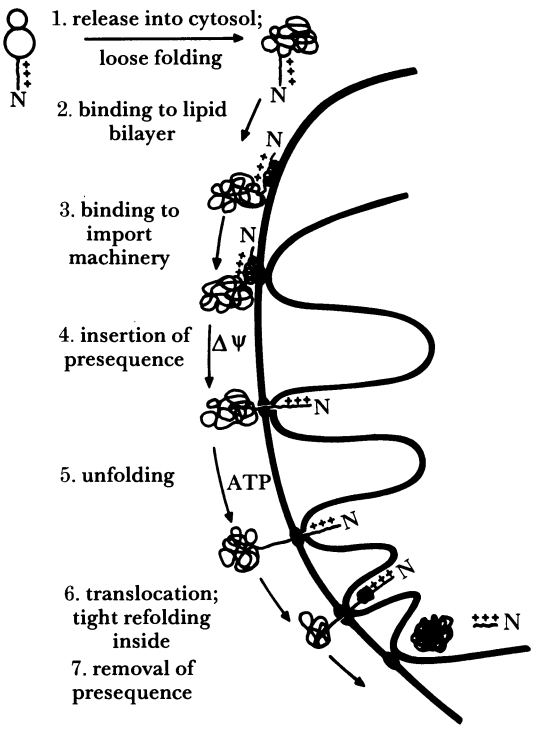

FIGURE 3. Suggested steps during post-translational import of a precursor protein into mitochondria. (From Verner & Schatz (1987).)

This incorrectly folded fusion protein could be imported by isolated mitochondria. Import was approximately 50-fold faster than that of the correctly folded protein and could still be detected at temperatures as low as 0–6 °C; at these low temperatures, the correctly folded precursor is no longer imported. Surprisingly, however, ATP stimulated import of the misfolded precursor as much as that of the correctly folded protein. This suggests that only stably unfolded molecules (such as nascent chains) do not require ATP; we speculate that ATP may be needed to drive unfolding to completion during import.

Remarkably, the misfolded precursor appeared to 'renature' on import into mitochondria: it regained trypsin-resistance and the ability to bind methotrexate. Also, the attached presequence was removed by the matrix protease which had failed to act on the misfolded protein outside the mitochondria. Removal of the presequence did not, by itself, trigger refolding as refolding was still observed on import into mitochondria from the mas1 mutant (Yaffe & Schatz 1984; Yaffe *et al.* 1985) which are deficient in the matrix protease (not shown).

These observations suggest a model (figure 3) according to which protein translocation across mitochondrial membranes is 'driven' by the free energy of refolding inside the mitochondria. Although we have evidence that a misfolded protein does indeed refold on import, direct evidence for protein unfolding outside the mitochondria is still lacking. Experiments to detect such an unfolding are currently being done.

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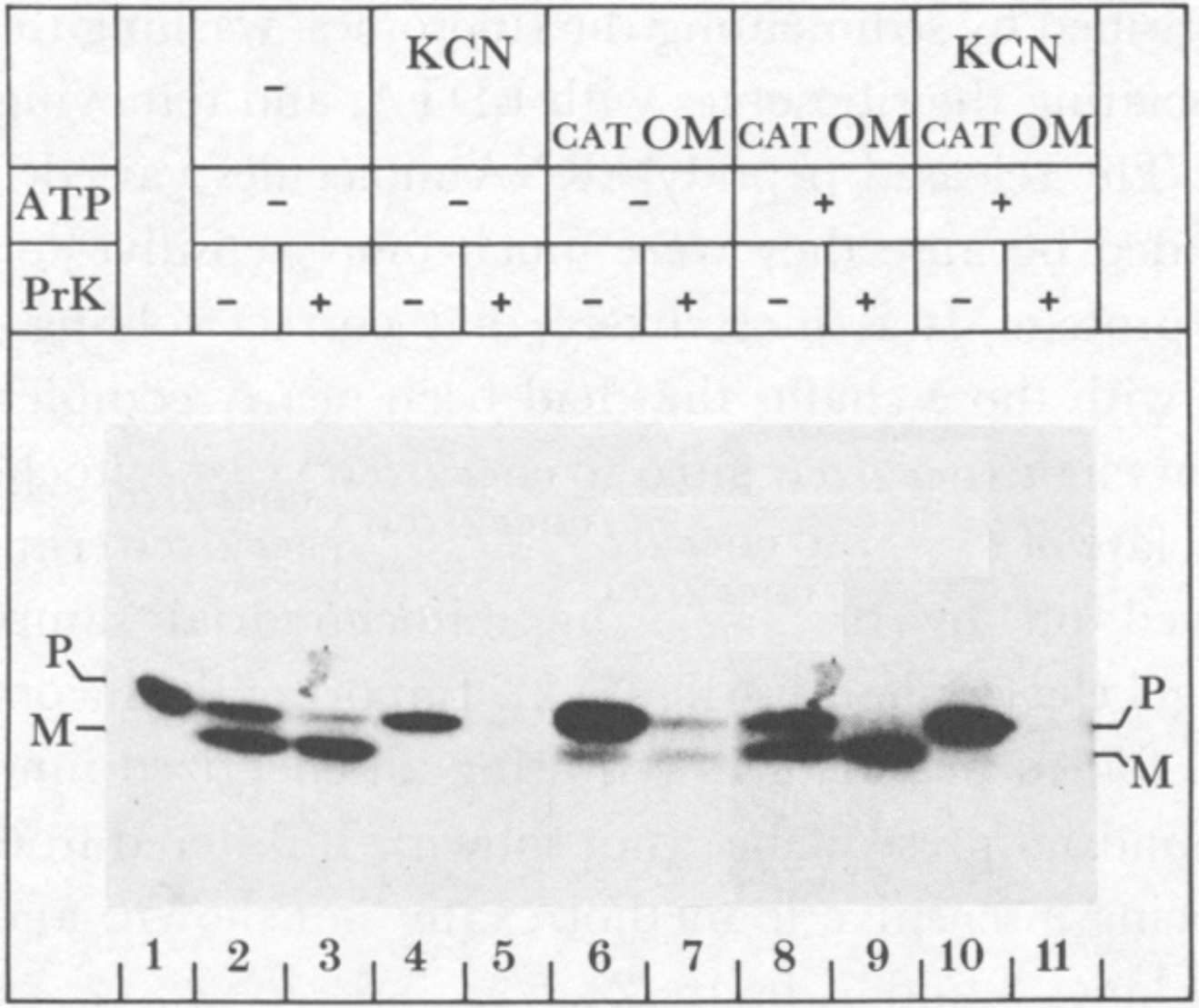

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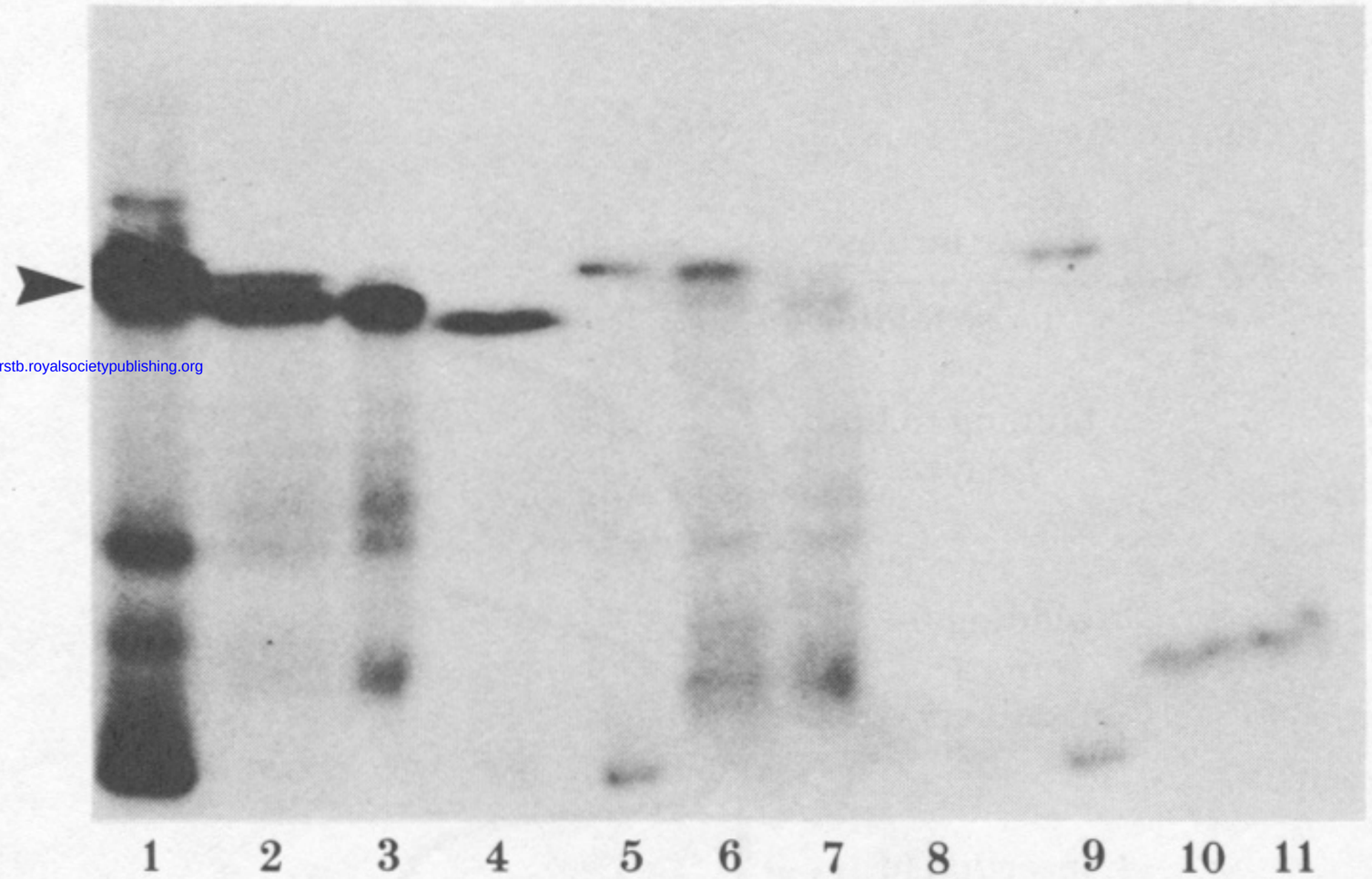

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