Modified precursor proteins as tools to study protein import into mitochondria

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The organization of cells in different compartments demands specific mechanisms to supply each organelle with its proper subset of proteins. While it is already known that presequences in precursor proteins contain the information which guides these proteins to their correct organelle, very little is known about the mechanism of the transmembrane movement of proteins.

We have studied the mechanism of protein transport into mitochondria using an artificial mitochondrial precursor protein containing the cytosolic enzyme mouse dihydrofolate reductase (DHFR) fused to the presequence of yeast cytochrome oxidase subunit IV (coxIV). This precursor (coxIV-DHFR) can be imported into mitochondria in vitro as well as in vivo [1] and can be purified in its native form after over-expression in Escherichia coli[2].

This precursor was modified so that it would get stuck in the import pathway and thereby provide a tool to identify molecular components of the translocation machinery. Using the same approach, we constructed a variety of chemically modified precursors which were used to analyse the tolerance of the mitochondrial translocational machinery for different chemical structures.

It has been well documented that the tertiary structure of a precursor protein is unfolded before or during the translocation process [2–6]. This implies that a stably folded protein with internal covalent cross-links such as disulphide bridges should not be importable into mitochondria. Such a protein coupled to the C-terminus of coxIV-DHFR should block the completion of the transport process, allowing only the DHFR moiety to pass into the matrix. The result would be a translocation intermediate representing an anchor that could be used to identify molecular components of the still hypothetical translocation machinery. We generated chimeric precursor proteins by chemically coupling appropriate passenger proteins to the C-terminus of coxIV-DHFR.

To this end, a unique cysteine group was introduced at the C-terminus of coxIV-DHFR while two internal cysteines were replaced by serines, using oligonucleotide-directed mutagenesis. Although these mutations destabilized the precursor protein, it could still be purified with high yield after over-expression in E. coli. Bovine pancreatic trypsin inhibitor (BPTI) was coupled via a bifunctional crosslinker to the C-terminal cysteine of the modified coxIV-DHFR. When this chimeric precursor was imported by isolated yeast mitochondria, it became stuck across the mitochondrial membrane, while the BPTI moiety was still accessible from outside for a membrane-impermeable reagent. The translocation intermediate could be generated in sufficient amounts to block the subsequent import of several authentic precursor proteins. This allowed, for the first time, the demonstration that mitochondria contain only a limited number (10−10) of import sites which can be shared by several authentic precursor proteins during their import into the organelle [7]. This translocation intermediate was used in photo-crosslinking experiments to identify proteins which are in close contact with the transmembrane precursor.

Since the precursor was stuck across the membrane with the DHFR moiety completely protease-protected and the BPTI moiety still accessible from outside for a membrane-impermeable reagent, we identified a single photo-crosslinked product which was generated in the light reaction with relatively high yield. At present this cross-linked mitochondrial protein is being studied in more detail.

The mutated coxIV–DHFR protein was further used to study the tolerance of the translocation process for different chemical structures. It was possible to import a membrane-impermeable, doubly negatively charged fluorescent dye that was coupled to the C-terminus of coxIV-DHFR [8]. This argues for a hydrophilic character of the transport channel. Surprisingly, even horse heart cytochrome c coupled via an internal lysine to coxIV–DHFR could be imported into the mitochondrial matrix [8]. This demonstrates that branched polypeptide chains can pass the transport channel which makes it unlikely that a polypeptide chain is moved across the membrane by specific recognition of peptide bonds and ratchet-like transposition of these bonds along a fully extended polypeptide chain. The covalently attached haem group of cytochrome c was most likely also imported which sets a new limit for the size of the proposed hydrophilic channel.

Abbreviations used: DHFR, dihydrofolate reductase; BPTI, bovine pancreatic trypsin inhibitor.
A similar technical approach was used to test whether coxIV-DHFR could also carry a nucleic acid into the mitochondrial matrix. There is indirect evidence that in some species RNA molecules are transported into mitochondria. The mitochondrial genomes of some organisms appear to lack some (Chlamydomonas, Tetrahymena), or even all (trypanosomatid protozoa), tRNA genes. Furthermore, two RNA-processing enzymes of mammalian mitochondria contain a nuclear-encoded RNA component essential for their activity. One of them is a site-specific endonuclease in mouse mitochondria [9], the other is a mitochondrial RNAaseP identified in HeLa cells [10]. The mechanism by which RNAs are imported into mitochondria is unknown. It may involve association of the RNA with a protein. To test whether the normal protein import pathway allows the passage of nucleic acids, the mutated coxIV-DHFR precursor protein was used as a vehicle to transport an oligonucleotide of 24 bases in length.

With the help of an oligonucleotide synthesizer, an aliphatic NH2-group was linked with a spacer of two CH2-groups to the 5'-PO4 of the oligonucleotide. This amino group was coupled to a bifunctional cross-linker the second functional group of which could react with the C-terminal SH-group of the mutated coxIV-DHFR protein. We could show that the precursor protein does indeed ‘pull’ the oligonucleotide into the mitochondrial matrix [11]. This shows that the protein import pathway tolerates the passage of such highly charged structures like a nucleic acid chain. Furthermore, the double-stranded version of the DNA-protein adduct was also imported with similar efficiency [11]. Since the double stranded 24-mer would be expected to have a diameter of 2 nm and form two complete helical turns, these experiments re-emphasize the fact that the mitochondrial protein import machinery is not restricted to the transport of fully extended, linear polypeptide chains.


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Protein secretion in Escherichia coli with particular reference to haemolysin

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Introduction

Gram-negative bacteria possess a complex envelope structure composed of an inner or cytoplasmic membrane, a rigid molecular sheath, the peptidoglycan layer, closely linked to the outer membrane surface layer. In Escherichia coli, the cell envelope in addition to phospholipids, lipopolysaccharide and peptidoglycan contains 10-15% of the cell's total cellular protein. This is distributed approximately equally between the inner and outer membranes and the periplasm, a hydrophilic gel-like compartment between the two membranes. The correct partitioning or targeting of this large amount of cellular protein to each separate layer or compartment of the cell envelope is therefore a highly complex and important process. This article will briefly outline the process of export from the cytoplasm, apparently common to all envelope proteins, and then consider in some detail the highly specialized system, with several novel features, which leads to the translocation (secretion) of haemolysin across both surface membranes to the medium.

Export of proteins into the cell envelope

Essentially all outer membrane and periplasmic proteins and a number of inner membrane proteins are initially synthesized with an N-terminal signal sequence which is cleaved by signal peptidase following translocation across the inner membrane. Specific partitioning signals which allow subsequent sorting of outer membrane and periplasmic proteins have not been identified, but some evidence indicates that the overall tertiary structure of a given protein finally determines its ability to assemble into the outer membrane [1]. In contrast, inner membrane proteins are retained in the inner membrane by hydrophobic anchors or specialized C-terminal signals [2, 3].

Several proteins including SecA, B, D, E and Y have been identified as essential for protein export in E. coli [4, 5]. SecA, a cytoplasmic protein, with affinity for the inner membrane, appears to be involved in an early stage in transport, involving recognition of the signal sequence and the consequent maintenance of envelope proteins in translocationally competent forms (see [6]). Translocation competence appears to be associated with some degree of unfolding (i.e. enhanced protease sensitivity) and other proteins, including SecB and trigger factor, a ribosome-associated protein, have also been implicated in the formation of unfolded proteins competent for translocation across the membrane [7-9]. In E. coli, however, no evidence for a specific ribonucleoprotein complex analogous to the eukaryotic SRP (signal recognition particle) has yet been obtained.

SecY is an integral membrane protein essential for protein translocation through the inner membrane in E. coli. SecY is a polypeptide of 49 kDa which appears to fold 10-times through the membrane [10] and acts at a late stage in the translocation process [6]. However, it is not clear whether SecY participates directly in the export process as a translocator or as a docking system which recognizes nascent polypeptide–SecA complexes before the final translocation step. However, evidence has been presented indicating that the signal sequence does interact with SecY [4].

Studies in vitro and in vivo have indicated that ATP and probably an energized membrane are both required for efficient protein export in E. coli, but no ATPase, specific for the transport process, has been identified [11]. Such energy requirements could be involved in further unfolding of the exported polypeptide or in the actual translocation through the lipid bilayer.

1989