Transport of Mitochondrial Precursor Proteins: Their Conformational Competence and Related Factors

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Most mitochondrial proteins are encoded by nuclear genes and translated in the cytosol as larger precursors containing NH₂-terminal cleavable extrapeptides known as presequences. The precursors are synthesized on free polysomes and released into a cytosolic pool, and then transported into the mitochondria. The half life of the precursors is less than several minutes. During or soon after the transport, the precursors are processed to respective mature enzymes by a matrix-localized processing protease. Recent studies revealed pertinent information on the nature and function of these transient presequences. They comprise 20–60 amino acid residues, are rich in positively charged amino acids, are devoid of acidic residues, and have no stretch of hydrophobic amino acids. The potential to form an amphiphilic helix is a property shared by many presequences, but as there is no direct evidence that such a structure is necessary for uptake by the mitochondria, its importance has been questioned. Gene fusion experiments have shown that the NH₂-terminal presequence is necessary, and in most cases, sufficient for targeting and import of the fusion protein. In these experiments, fusion proteins consisting of the presequence of a mitochondrial precursor protein fused to the NH₂-terminus of a “passenger” protein, are often efficiently imported into the mitochondria *in vitro* or *in vivo*. The passenger protein can be a mitochondrial protein lacking its own presequence or a protein not normally imported into the mitochondria (see Refs. 1 and 2 for reviews).

Transport of proteins into the mitochondria is post-translational. The precursor proteins remain in the cytosol in a completed form for a few to several minutes until imported into the organelle. The precursors bind to the outer surface of the mitochondria, probably to the “receptor”, and transverse the mitochondrial membranes. The precursors are then transported across the membranes presumably in an unfolded state. Therefore, the cytosolically released precursor proteins must be unfolded or be maintained in an unfolded conformation prior to import (see below). In 1981, we showed that the *in vitro* synthesized precursors for mitochondrial carbamyl phosphate synthetase and ornithine transcarbamylase (OTC) have aggregation states and conformations different from those of the respective mature enzymes (3), thus suggesting that the presequences affect conformations of these proteins directly or indirectly. However, molecular details and the significance of our findings remained to be determined. This minireview deals with recent progress in in-

vestigations on the conformation and transport competence of mitochondrial precursor proteins, and their interaction with cytosolic protein factors. Our model that the presequence-specific cytosolic factor (referred to as the presequence binding factor or PBF) binds to the precursor proteins, prevents their folding and maintains them in a transport-competent conformation, is presented.

Mitochondrial Precursor Proteins Are Unfolded During Translocation. Several lines of evidence indicate that precursor proteins cannot be transported into the mitochondria in a tightly folded state. This topic was recently reviewed by Eilers and Schatz (4) and by Meyer (5), and is briefly summarized here. First, precursor proteins of *Neurospora crassa* ATPase $F_1\beta$ subunit and cytochrome c_1 could be trapped as translocation intermediates when *in vitro* transport was performed at low temperature or after binding of the precursor proteins to antibodies; the NH_2 -terminal portion of which had already penetrated across the two mitochondrial membranes, whereas the remainder of the precursor polypeptide remained exposed on the mitochondrial surface. Second, methotrexate, a folate analogue, was found to block mitochondrial import of a purified artificial precursor protein consisting of mouse dihydrofolate reductase (DHFR) fused to the presequence of yeast cytochrome oxidase subunit IV; methotrexate is known to inhibit unfolding of DHFR. This folate analogue did not inhibit import of the authentic precursor of cytochrome oxidase subunit IV. Third, when the purified artificial precursor protein was denatured in 8 M urea, the denatured precursor protein was imported much more efficiently into isolated mitochondria than the native precursor protein. Fourth, when conformation of the artificial precursor protein was destabilized by point mutations, import into the mitochondria was enhanced. All these observations mean that unfolding is necessary for translocation across mitochondrial membranes.

Presequence Does Not Prevent Folding of the Precursor Protein. One of the functions of the presequence might be to prevent or retard folding of the mature portion of mitochondrial precursor proteins and to maintain them in a transport-competent conformation. To analyze the properties and conformation of the precursor proteins, they have to be isolated in a homogeneous and functional state. However, isolation of the precursor proteins from eukaryotic cells is difficult, as the steady state concentrations are extremely low. Therefore, attempts have been made to express precursor proteins in *Escherichia coli*. Schatz and his colleagues purified a recombinant fusion protein consisting of the presequence of yeast cytochrome oxidase subunit IV to the NH_2 -terminus of mouse DHFR, and used this artificial precursor protein to study mitochondrial protein import (4). The purified soluble fusion protein exhibits DHFR activity, and K_m values of the fusion protein for NADPH and dihydrofolate are similar to those of authentic mouse DHFR. Endo and Schatz (6) showed that the fusion precursor protein closely resembles authentic mouse DHFR with respect to the secondary structure (measured by CD spectra) and stability towards urea (measured by tryptophan fluorescence and enzyme activity). These results indicate that, with regard to this artificial precursor, the presequence does not significantly alter the folding of the attached passenger protein.

To determine whether such is the case for authentic precursor proteins, we synthesized the precursor (7) and mature form of rat OTC in *E. coli*. Both the recombinant precursor and mature OTCs formed inclusion bodies and represented 5–10% of the total bacterial protein. Both forms were extracted from the inclusion bodies with 8 M urea or 6 M guanidine·HCl and were obtained in an essentially

homogeneous state, as judged by SDS-polyacrylamide gel electrophoresis. When guanidine-denatured mature OTC was diluted and incubated at 0°C for 40–60 h, it was reactivated to a specific activity about 20% of that of the purified native enzyme. Guanidine-denatured precursor form of OTC was activated to a similar extent under similar conditions, although activation kinetics differed somewhat from that of the mature OTC (unpublished). These results and sucrose gradient centrifugation analysis showed that the presequence does not prevent the OTC precursor from folding into an enzymatically active trimeric form. Most recently, the precursor of rat mitochondrial aspartate aminotransferase was synthesized in *E. coli* and isolated in the form of an enzymatically active dimeric protein (8). All these results indicate that the presequence portion does not always interfere with much of the folding of the mature portion, even for natural precursor proteins.

More Than One Cytosolic Factor Is Required for Protein Import. It is thus assumed that there is a component(s) in the cytosol, the function of which is to maintain completed precursor proteins in a conformation enabling transport across mitochondrial membranes. In 1983, we found that a protein component(s) in the reticulocyte lysate markedly stimulates the uptake of *in vitro*-synthesized OTC precursor by isolated mitochondria (9). However, purification and characterization of this factor(s) was hampered by the fact that the reticulocyte lysate used in the synthesis of the precursor protein was inevitably present in the *in vitro* import assay. Ohta and Schatz (10) reconstituted an *in vitro* import system which included the purified precursor for the yeast mitochondrial ATPase β -subunit, and showed that a yeast cytosolic protein factor is required for import. The apparent molecular weight was estimated to be about 40,000.

Deshaies *et al.* (11) showed that 70 kDa heat shock proteins (hsp70) of yeast are involved in the post-translational import of precursor polypeptides into both the mitochondria and lumen of the endoplasmic reticulum. Yeast cells depleted of a subset of hsp70-like polypeptides contained unprocessed precursors of proteins destined for mitochondria (F_1 -ATPase β -subunit) or the endoplasmic reticulum (prepro- α -factor and preprocarboxypeptidase Y). Murakami *et al.* (12) developed an *in vitro* import system which contains a precursor protein synthesized in a wheat germ cell-free system and is dependent on the addition of yeast postmitochondrial supernatant. They showed that the import stimulatory activity consists of at least two distinct factors. One is hsp70 and the other is an *N*-ethylmaleimide-sensitive protein(s).

A variety of functions have been postulated for hsp70s. A member of the hsp70 protein family catalyzes an ATP-dependent depolymerization of clathrin from coated vesicles *in vitro*. An hsp70-like protein associates with immunoglobulin heavy chains in IgG immunoprecipitates, and this association is disrupted by ATP. The major heat-inducible hsp70, tightly bound to the nucleolus, is released by ATP. These properties suggest that hsp70s are involved in the disruption or dissociation of multi-subunit complexes or denatured aggregates in an ATP-dependent manner. A simple extension of this suggestion is that an hsp70-related protein(s) is involved in the relaxation of the tertiary structure within a single polypeptide of mitochondrial precursor proteins. However, the hsp70(s) is apparently not specific for mitochondrial precursor proteins, because it stimulates translocation of both mitochondrial and secretory precursor polypeptides. Biochemical studies revealed that a mitochondrial presequence-specific cytosolic factor is also involved in mitochondrial protein import.

Presequence Binding Factor (PBF) Is Involved in Protein Import. We established an *in vitro* import system in which the purified recombinant OTC precursor was imported into isolated mitochondria (7). Using this system, we confirmed our previous findings (9) that a cytosolic factor(s) is required for import of the precursor. This factor forms a soluble complex with the recombinant OTC precursor. We purified this protein factor from rabbit reticulocyte lysate about 90,000-fold by affinity chromatography using the OTC precursor as a ligand (unpublished results). A similar protein factor was also purified from the cytosol fraction of rat liver and heart. ATP has no effect on the complex formation between the purified factor and the purified precursor. These results indicate that ATP is not involved either in formation of the complex or in its dissociation. Formation of this complex is effectively inhibited by micromolar concentrations of the synthetic presequence of the OTC precursor. We tentatively refer to this factor as "presequence binding factor (PBF)".

Ono and Tuboi (13) developed a unique *in vitro* import system in which the chemically synthesized presequence (34 amino acid residues) of ornithine aminotransferase was used instead of natural precursor proteins. The import of the presequence peptide was energy-dependent and thus mimicked the import of natural precursor proteins. They showed that a cytosolic factor present in the reticulocyte lysate stimulates the import of the presequence. They purified the factor about 20,000-fold. Molecular weight of this factor was estimated by gel filtration to be 200,000. This cytosolic factor and the synthetic presequence were shown to form a complex. It remains to be elucidated whether this factor is identical to PBF.

We propose that the binding of PBF to the precursor proteins is the first step in multistep reactions of mitochondrial protein import. PBF presumably binds to the presequence portion of the nascent precursor protein, prevents it from folding and holds it in an import-competent form. Hsp70 may also be involved in the relaxation of the precursor polypeptide. The precursor-PBF complex may then bind to the mitochondrial surface, and the presequence portion of the precursor polypeptide may be displaced from PBF to the "receptor" on the mitochondrial surface, after which the precursor is imported into the organelle. The precursor-PBF complex may have a higher affinity for the "receptor" than the precursor protein alone. Isolation of the homogeneous PBF should facilitate studies on targeting and translocation of mitochondrial precursor proteins and for identification of the "receptor" for precursor protein.

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