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Anion Carriers of Mitochondrial Membranes

With 147 Figures

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Biogenesis of Mitochondrial Proteins

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The majority of mitochondrial proteins are encoded by nuclear genes and are imported into the organelle after being synthesized on cytoplasmic ribosomes. During the last years our knowledge of the different stages of this import process of proteins into mitochondria has increased greatly.

Most of the precursor proteins are synthesized with a transient amino-terminal extension ("presequence") which is removed in the mitochondrial matrix. On the other hand, several precursors (*e.g.* outer membrane proteins like porin or inner membrane proteins like the ADP/ATP carrier and the uncoupling protein of brown adipose tissue) are synthesized without a cleavable presequence. The import of almost all precursor proteins depends on the presence of ATP. ATP is thought to mediate the unfolding of the precursor proteins *via* cytosolic factors (unfoldases). Specific proteinaceous receptors on the mitochondrial surface recognize precursor proteins and deliver them to a general insertion protein (GIP) in the outer membrane. Further import occurs at translocation contact sites between outer and inner membrane which form a hydrophilic environment for the translocation of the precursor proteins. The insertion of precursor proteins into translocation contact sites requires a membrane potential. The completion of precursor translocation into the inner membrane or matrix is independent of the membrane potential. In the mitochondrial matrix, the amino-terminal presequences are removed by the matrix processing peptidase (MPP). MPP is a soluble protein of 57 kDa in *Neurospora crassa*. A second protein of 52 kDa, the processing enhancing protein (PEP; equivalent to the *mas1* gene product in yeast),

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teins *e.g.* porin and the intermembrane space protein cytochrome c (for review see Nicholson & Neupert, 1988).

In the last two years, it was demonstrated that also nucleoside triphosphates (NTPs: ATP or GTP) are needed for the import of proteins into mitochondria (Pfanner & Neupert, 1986; Pfanner *et al.*, 1987a; Chen & Douglas, 1987a; Eilers *et al.*, 1987; Hartl *et al.*, 1987a). It has been suggested that NTPs maintain or confer an import-competent conformation in mitochondrial precursor proteins. This is supported by experiments where the proteolytic sensitivity of precursor proteins is increased in the presence of NTPs (Pfanner *et al.*, 1987a; Verner & Schatz, 1987), indicating that a less folded conformation is sustained by NTP hydrolysis and that such a conformation is necessary for import. Conformational alteration of a precursor protein (porin) can substitute for the ATP requirement (Pfanner *et al.*, 1988b). The levels of NTPs required depend primarily on the mature part of the precursor protein. For example, precursors having identical presequences but different mature polypeptides require different concentrations of NTPs for optimal import (Pfanner *et al.*, 1987a). It appears that NTPs are necessary for conferring import-competence during all steps that precede and include the interaction of the precursor with the outer membrane (Pfanner & Neupert, 1987; Eilers *et al.*, 1987). Eilers & Schatz (1986) showed that a stable tertiary structure of a protein is incompatible with import of the protein into mitochondria. Methotrexate, an inhibitor of dihydrofolatereductase (DHFR), blocked import into mitochondria of a fusion protein between a mitochondrial presequence and DHFR, most probably by imposing a defined, stable, tertiary structure. A similar result was obtained with a fusion protein between the β-subunit of F_1-ATPase and copper metallothionein (Chen & Douglas, 1987b).

SPECIFIC RECOGNITION AND MEMBRANE INSERTION OF PRECURSOR PROTEINS

Receptors

The targeting informations contained in the precursor proteins for specific mitochondrial recognition and sorting have to be decoded by components of the mitochondrial import machinery. One type of component of this import machinery are import receptors, which are of a proteinaceous nature.

Proteinaceous receptors on the outer surface of mitochondrial membranes were first demonstrated by shaving isolated mitochondria with low concentrations of proteases which do not penetrate or destroy the outer membrane (Gasser *et al.*, 1982; Riezmann *et al.*, 1983; Zwizinski *et al.*, 1984; Pfaller & Neupert, 1987; Kleene *et al.*, 1987). Following this treatment, specific binding of precursor proteins to the outer

membrane was blocked and import was greatly reduced (see also below: bypass import).

Binding of precursor proteins to receptors can be stalled either by disrupting the membrane potential, low temperature, or in the special case of apocytochrome c by deuterohemin. Deuterohemin is a heme analogue which prevents covalent attachment of heme to the precursor apocytochrome c and thus prevents subsequent translocation across the outer membrane (Hennig & Neupert, 1981). Under these conditions, apocytochrome c could still bind to mitochondria independently of import. When the inhibition by deuterohemin was reversed by adding excess amounts of hemin, apocytochrome c was subsequently imported from its receptor sites into the intermembrane space (Hennig & Neupert, 1981; Hennig *et al.*, 1983).

How many different receptors exist on the mitochondrial surface to mediate recognition and binding of the many different precursor protein having or having not cleavable presequences? We postulated at least three different types of receptors (Pfaller *et al.*, 1988a). This is based on different sensitivity of the receptors to trypsin and elastase treatment. Import of a number of different precursor proteins is greatly reduced by treatment of mitochondria with low concentrations of elastase (examples include ADP/ATP carrier and porin). Import of the β-subunit of F_1-ATPase, however, is not significantly affected by pretreatment of elastase (up to 10 μg/ml; Zwizinski *et al.*, 1984; Pfaller *et al.*, 1988a). In contrast to this, import of all these precursors is sensitive to pretreatment of mitochondria with trypsin. This suggests that at least two different types of receptors exist. Since the precursor of porin does not compete with the ADP/ATP carrier for binding to its receptor, at least three types of receptors can be postulated (one for porin, one for ADP/ATP carrier and one for F_1-β subunit). All three receptors are thought to deliver the bound precursor proteins to a general insertion protein (GIP) in the outer membrane (see below). A fourth type of receptor is the apocytochrome c receptor (Hennig & Neupert, 1981; Hennig *et al.*, 1983). Binding of apocytochrome c to the mitochondrial surface can be blocked only by pretreatment of mitochondria with high concentrations of trypsin (> 40 μg/ml). Recent results (Nicholson *et al.*, 1988) indicate that the enzyme cytochrome c-heme lyase, which is located on the inner side of the outer membrane (therefore being not easily protease-accessible), could be the receptor for apocytochrome c. Apocytochrome c is able to spontaneously insert into lipid bilayers in a nonspecific manner with low affinity (Rietveld *et al.*, 1983, 1985, 1986a,b; Rietveld & Kruijff, 1984; Dumont & Richards, 1984) and could thereby expose domains to the binding protein, cytochrome c-heme lyase. Cytochrome c seems to have a unique import pathway different from all other precursor proteins (for review see Nicholson & Neupert, 1988).

General insertion protein (GIP)

Competition experiments of precursor proteins for components of the mitochondrial import machinery are possible when precursor proteins are available in sufficient chemical amounts. This was achieved either by expression in yeast (Ohta & Schatz, 1984) or *E.coli* (Eilers *et al.*, 1987). Another approach was to isolate a mitochondrial protein without a transient presequence and alter its conformation to that of its precursor form. We isolated porin from the outer mitochondrial membrane of *Neurospora crassa* and converted it to a water-soluble form (ws-porin) which behaves in many respects like the authentic biosynthetic porin precursor (Pfaller *et al.*, 1985). Ws-porin was shown to compete for the import of precursors destined for the three other mitochondrial compartments: the Fe/S protein of the bc_1-complex (intermembrane space), the ADP/ATP carrier (inner membrane), subunit 9 of F_o-ATPase (inner membrane) and subunit β of the F_1-ATPase (matrix). Competition does not occur at the level of the receptor proteins but at a common site at which precursors are inserted into the outer membrane. We suggest that distinct receptor proteins recognize precursor proteins and transfer them to a general insertion protein (GIP) in the outer membrane. Beyond GIP, the import pathways diverge, either to the outer membrane (*e.g.* porin) or to translocation contact-sites and then subsequently to the other mitochondrial compartments (Pfaller *et al.*, 1988a).

Bypass import

Proteolytic degradation of receptor sites on the mitochondrial surface strongly reduces the efficiency of mitochondrial protein import (up to 80 - 95%; see also above). The remaining residual import (bypass import) still involves basic mechanisms of protein import, including: insertion of precursors into the outer membrane, requirement for ATP and a membrane potential, and translocation through contact sites between both mitochondrial membranes. The import of a chloroplast protein (small subunit of ribulose-1,5-biphosphate carboxylase/oxygenase) into isolated mitochondria which occurs with a low rate is not inhibited by a protease-pretreatment of mitochondria, indicating that this precursor only follows the bypass pathway. The low efficiency of bypass import suggests that this unspecific import does not disturb the uniqueness of mitochondrial protein composition. We conclude that mitochondrial protein import involves a series of steps in which receptors sites appear to be responsible for the specificity of protein uptake (Pfaller *et al.*, 1988b).

TRANSLOCATION CONTACT SITES

Proteins which are imported into the mitochondrial matrix or inner membrane must cross two membranes barriers to reach their final location. It has been demonstrated for a variety of proteins that import occurs at sites where inner and outer membrane come close enough to be spanned and crossed in a single event. Involvement of translocation subunit and cytochrome c_1 (Schleyer & Neupert, 1985), the Fe/S protein of bc_1 complex (Hartl *et al.*, 1986), the ADP/ATP carrier (Pfanner & Neupert, 1987), cytochrome b_2 (Pfanner *et al.*, 1987c; Hartl *et al.*, 1987b) and a number of fusion proteins. Three distinct methods yielded translocation intermediates spanning both membranes: import at low temperature, pre-binding of antibodies to carboxy-terminal precursor portions (Schleyer & Neupert, 1985) and import at low levels of NTPs (Pfanner *et al.*, 1987a,c). Precursors were thereby trapped in an intermediate position with the amino-terminal presequence in the mitochondrial matrix and other, probably carboxy-terminal, portions of the precursors outside the outer membrane. The topology of the intermediates was examined by their accessibility to the matrix-processing peptidase (see below), and to externally added proteases and antibodies (Söllner *et al.*, 1988). Immunochemical studies (labelling the contact site intermediates with protein A-gold particles *via* the bound antibodies) demonstrated the identity of morphologically described (Hackenbrock, 1968) and the biochemically defined contact sites (Schwaiger *et al.*, 1987). Contact sites appear to be stable structures which can be enriched after subfractionation of mitochondria by sonication (Schwaiger *et al.*, 1987). Contact site intermediates could be extracted from the membranes with hydrophilic perturbants, such as urea or at alkaline pH, suggesting that mitochondrial precursor proteins are imported through a hydrophilic membrane environment (Pfanner *et al.*, 1987c). Specific proteins in contact sites are probably involved in constituting the architecture of these sites and participate in protein translocation.

Mitochondrial processing peptidase

During or shortly following the translocation step, the amino-terminal presequences of many proteins directed to the inner membrane or matrix are removed by a specific protease which is located in the matrix (Böhni *et al.*, 1980; Mori *et al.*, 1980; Conboy *et al.*, 1982; McAda & Douglas, 1982; Miura *et al.*, 1982; Böhni *et al.*, 1983; Schmidt *et al.*, 1984; Miura *et al.*, 1986). This occurs very rapidly *in vivo*. Processing, however, is not obligatory for import since the precursors to subunits β and IX of F_0F_1-ATPase could be imported into mitochondria when proteolytic processing was blocked by o-phenanthroline (Zwizinski & Neupert, 1983). Similarly, the precursor to the Fe/S

protein of the bc_1 complex could be imported and accumulated in the matrix when processing was blocked (Hartl *et al.*, 1986).

The matrix-located peptidase has been purified to homogeneity from *N.crassa*. The purification was about 10,000 fold (starting with a cell extract) and yielded two bands on SDS-PAGE (PEP: 52 kDa and MPP: 57 kDa; Hawlitschek *et al.*, 1988). The matrix processing peptidase (MPP: 57 kDa) has a low intrinsic enzyme activity in the absence of the processing enhancing protein (PEP: 52 kDa) and the latter has a strong stimulating influence on the processing, being by itself completely inactive. We have cloned the cDNAs for both proteins (Hawlitschek *et al.*, 1988; H. Schneider *et al.*, in preparation). The amino acid sequence of *N.crassa* PEP shows 60 % homology to the *mas1* gene product from the yeast *S.cerevisiae*. This suggests that *mas1* encodes the yeast equivalent to *N.crassa* PEP. *N.crassa* MPP (H. Schneider *et al.*, in preparation) has a high degree of homology to the protein encoded the *mas2* gene of *S.cerevisiae* (Yaffe & Schatz, 1984; Yaffe *et al.*, 1985), which is equivalent to the *mif2* gene described by Pollock *et al.* (1988). Interestingly, the two cooperating components MPP and PEP are structurally related, suggesting that the respective genes are of common evolutionary origin (Pollock *et al.*, 1988).

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