BIOCHEMICAL SOCIETY TRANSACTIONS

London: The Biochemical Society © 1987

Managing Editor D. C. WATTS Book Reviews Editor B. MORELAND

Contents Volume 15, Part 1, February 1987

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Translocation intermediates on the import pathway of proteins into mitochondria

FRANZ-ULRICH HARTL, NIKOLAUS PFANNER and WALTER NEUPERT

Institut für Physiologische Chemie der Universität München, Goethestr. 33, 8000 München 2, Federal Republic of Germany

Import of cytoplasmically made precursor proteins into mitochondria can be subdivided into the following two steps: (i) binding to receptors on the outer mitochondrial surface (Zwizinski *et al.*, 1984), (ii) translocation across the mitochondrial membranes (Schleyer & Neupert, 1985), (iii) processing of precursors, in the case of those synthesized with *N*-terminal extensions, by the matrix-located peptidase (Böhni *et al.*, 1980; Schmidt *et al.*, 1984), and, finally, (iv) assembly into biologically active protein complexes within the correct mitochondrial compartment (Schmidt *et al.*, 1983). Translocation across or into the inner membrane is dependent on a membrane potential ($\Delta\psi$) across the inner membrane (Schleyer *et al.*, 1982; Pfanner & Neupert, 1985).

It has recently been shown that, for precursors of subunit β of H^+-ATPase and of cytochrome c_1, import into mitochondria occurs via translocation contact sites between outer and inner membranes without release into the intermembrane space (Schleyer & Neupert, 1985). This is of special interest with regard to the intramitochondrial sorting of precursor proteins to their final locations in different mitochondrial compartments. For example, cytochrome c_1 is situated with a large hydrophilic domain in the intermembrane space anchored to the inner membrane, whereas subunit β of H^+-ATPase reaches a matrix location.

In the present report we will focus on the import pathways of two other mitochondrial precursor proteins, namely the Rieske Fe/S-protein of complex III (bc_1-complex) and the ADP/ATP-carrier of the inner membrane.

Import pathway of Fe/S-protein

Rieske Fe/S-protein (M_r 25000) is a peripheral component of complex III carrying a 2Fe–2S cluster. It protrudes into the intermembrane space with a large hydrophilic part of the molecule. Unlike cytochrome c_1, it does not appear to be firmly anchored in the inner membrane by transmembrane segment(s).

Precursor of Fe/S-protein is processed in two steps. Fe/S-protein is cytoplasmically synthesized as a larger precursor of M_r 28000 (p-Fe/S). In order to study import *in vitro*, mitochondria of *Neurospora crassa* were incubated in reticulocyte lysates programmed with *Neurospora* poly(A^+)-RNA in the presence of [^{35}S]methionine. Under these conditions, p-Fe/S was transferred into mitochondria and processed to the mature size (m-Fe/S). Addition of the metal chelators EDTA and 1,10-phenanthroline caused inhibition of the matrix-localized peptidase and lead to accumulation of p-Fe/S. Interestingly, 1,10-phenanthroline alone causes the accumulation of an intermediate sized species of Fe/S-protein of M_r 25000 (i-Fe/S). This i-Fe/S is not an artifact *in vitro*, since its transient appearance can also be demonstrated during pulse–chase experiments *in vivo*. Therefore, processing of p-Fe/S follows a two-step mechanism.

Import of p-Fe/S occurs via translocation contact sites. This could be shown by two different experimental approaches: appropriate amounts of antiserum, directed against Fe/S-protein, were added to reticulocyte lysates containing the labelled precursor. In the resulting antibody–Fe/S complexes, the *N*-terminus of the precursor was free to recognize the import machinery of mitochondria. When import was performed, p-Fe/S was partially translocated. This occurred in an energy-dependent manner and processing by the matrix peptidase was observed. The translocation intermediates were accessible to proteinase K added to the intact mitochondria, confirming that the antibody-bound protein was indeed spanning both outer and inner membranes, as has recently been shown for two other mitochondrial precursor proteins (Schleyer & Neupert, 1985). When, instead of antibody prebinding, import of Fe/S-protein was performed at lower temperatures (between 8 and 15°C), similar translocational intermediates could be accumulated which had reached the matrix space with their *N*-terminus (since they were processed by the matrix peptidase), but still had a major part of the polypeptide located outside the outer membrane (since they were digested by externally added protease).

In the absence of proteolytic processing precursor is imported into the matrix space. After inhibition of the matrix peptidase with chelating agents, p-Fe/S was translocated into a position resistant to added proteinase K. Titration with increasing amounts of protease revealed a high degree of protection. Further experiments were performed to determine the submitochondrial localization of imported p-Fe/S. Alkaline extraction of mitochondria, known to leave only proteins in the membrane fraction that are integrated into the phospholipid bilayer, caused extraction of all imported species of Fe/S-protein. Only precursor bound unspecifically to the outer surface of mitochondria was resistant to extraction. Also, the authentic m-Fe/S from mitochondria labelled *in vivo* was solubilized at alkaline pH. We also tested extractability with increasing salt concentrations. Imported p-Fe/S and i-Fe/S proved to be soluble in low salt, whereas m-Fe/S, *in vitro* and *in vivo*, were insoluble even in high salt. Therefore, p-Fe/S and i-Fe/S have to be regarded as water-soluble species, whereas m-Fe/S behaved like a peripheral membrane protein.

Based on these findings and on the fact that translocation of p-Fe/S into its protease-protected position is dependent on a membrane potential across the inner membrane, a location in the matrix compartment was assumed. Subfractionations of mitochondria with digitonin were performed

Abbreviations used: p-Fe/S, precursor of Fe/S-protein; m-Fe/S, mature Fe/S-protein; i-Fe/S, intermediate sized Fe/S protein.

to check this assumption with an independent method. Soluble components of intermembrane space and matrix were differentially released from the mitochondria. Imported p-Fe/S and i-Fe/S co-fractionated with fumarase, the marker for the matrix compartment. Adenylate kinase, the marker for the intermembrane space, was released at distinctly lower digitonin concentrations.

In conclusion, imported p-Fe/S and i-Fe/S are soluble, not membrane-bound, species in the import pathway; they are located in the matrix compartment.

Precursor imported into the matrix is on the correct assembly pathway. Several experiments were carried out to evaluate the physiological significance of matrix-localized p-Fe/S and i-Fe/S. Upon reactivation of processing peptidase by adding Mn^{2+}, the imported matrix-located precursor could be completely chased to the mature size. This processing occurred in the presence of inhibitors of oxidative phosphorylation and uncouplers (i.e. in the absence of a membrane potential). Pretreatment of mitochondria with protease, before activating the processing peptidase with Mn^{2+}, did not change this result. m-Fe/S chased from imported p-Fe/S was localized by digitonin fractionation and subsequent protease treatment. In digitonin-treated mitochondria with an opened intermembrane space, p-Fe/S was completely protected against added proteinase K. In contrast, most m-Fe/S was in a location accessible to protease. Digestion of m-Fe/S resulted in the formation of an approx. 24 000-M_r fragment that was further protected against cleavage. A fragment of the same size was also observed when the assembled Fe/S from labelled mitochondria *in vivo* was submitted to combined digitonin and protease treatment. From these data we conclude that imported m-Fe/S, processed from matrix-localized precursor, reaches a position at the outer surface of the inner membrane which is comparable with that of the authentic protein. In addition, at least partial assembly of imported m-Fe/S with other subunits of complex III could be demonstrated by co-precipitation of m-Fe/S with an antibody directed against cytochrome c_1 after solubilization with Triton X-100.

Import pathway of ADP/ATP-carrier

The precursor to the ADP/ATP-carrier is synthesized in the cytoplasm without a cleavable presequence. It can be bound to de-energized mitochondria (dissipated membrane potential) and imported from the binding sites after re-energization (re-established membrane potential) (Zwizinski *et al.*, 1983). ADP/ATP-carrier that has reached its final location in the inner mitochondrial membrane is able to bind the specific inhibitor carboxyatracyloside and to pass over hydroxyl-apatite (Schleyer & Neupert, 1984). The import pathway of ADP/ATP-carrier could be resolved into several distinct steps by investigating the association of precursor with de-energized and energized mitochondria.

In the absence of a membrane potential, precursor reaches a position past the receptor but before entering the inner membrane. Specific binding of the precursor to de-energized mitochondria at 25°C directed the precursor to a hydrophilic environment, as shown by solubility at alkaline pH. Import into the inner membrane from these sites was found to occur when the mitochondria were re-energized. Mitochondria were treated with protease under conditions which lead to loss of the ability to import free precursor (suggesting that all receptor sites were degraded). Most of the specifically associated precursor, however, was protected against protease digestion and could be imported into the inner membrane after re-establishing a membrane potential. We conclude that this translocation intermediate is past the initial precursor–receptor interaction, but before entrance into the inner membrane.

Precursor to ADP/ATP-carrier is imported via translocation contact sites. Precursor was incubated with energized mitochondria at various temperatures . At 25°C precursor was completely imported into the inner membrane. At 2 C it remained accessible to externally added protease. After raising the temperature to 25°C, however, it could be imported to its functional location even after dissipating the membrane potential. This suggests that part of the molecule had already interacted with the inner membrane while a major domain was still outside the mitochondria. Thus, precursor of ADP/ATP-carrier also appears to be imported through translocation contact sites between outer and inner membranes.

Conclusions

Precursor of Fe/S-protein of complex III (bc_1-complex), a protein with its final location at the outer surface of the inner membrane, is first completely translocated into the matrix compartment via translocation contact sites. There,

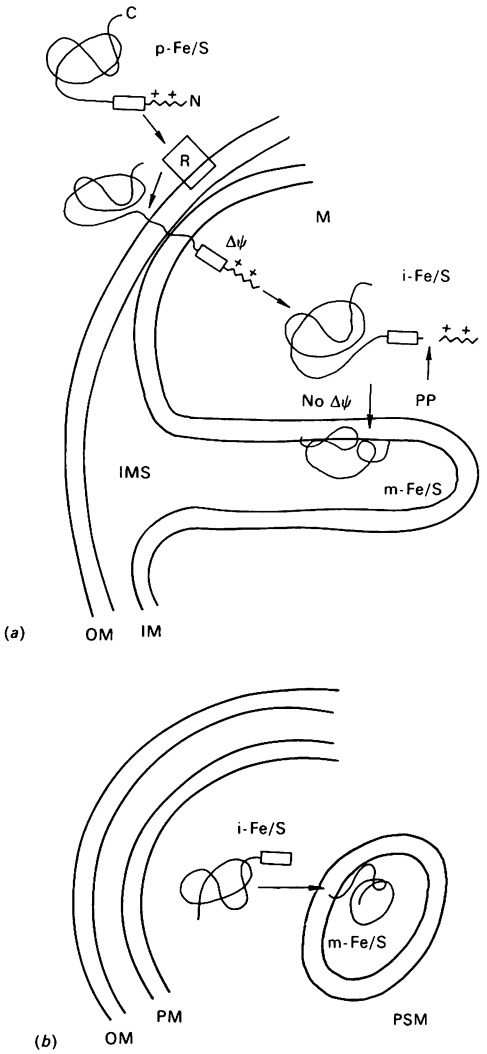

Fig. 1. *Hypothetical tranport and assembly pathways of Fe/S-proteins of mitochondrial and bacterial bc_1-complexes*

(*a*) Mitochondria; (*b*) *Rhodopseudomonas sphaeroides*. Abbreviations: OM, outer membrane; IMS, intermembrane space; IM, inner membrane; PM, plasma membrane; M, matrix; PSM, photosynthetic membrane; R, receptor; PP, processing peptidase; Δψ, membrane potential.

intermediate sized Fe/S is produced by the action of the matrix peptidase. This i-Fe/S then has the ability to re-cross the inner membrane, from the opposite direction, to finally become assembled with other subunits of complex III, especially with cytochrome c_1 (Fig. 1*a*). (It remains to be determined exactly when and where i-Fe/S is processed to m-Fe/S.)

We propose that this seemingly complicated assembly pathway can be satisfactorily explained in terms of the endosymbiotic origin of mitochondria. In prokaryotic cells, such as the photosynthetic bacterium *Rhodopseudomonas sphaeroides*, a membrane bound bc_1-complex (corresponding to the bc_1-complex in mitochondria) contains a Fe/S-protein highly homologous in structure, function and topology to the mitochondrial Fe/S-protein (Gabellini & Sebald, 1986). In the bacterium this protein is synthesized as a precursor about 1–2 kDa larger than the mature protein and is directed from the cytosol across the photosynthetic membrane (Fig. 1*b*). The endosymbiont hypothesis implies that after transfer of the gene to the nucleus of the host cell, the Fe/S-protein had to be translocated across both membranes back into the mitochondrial matrix. For this purpose, precursor proteins have acquired positively charged presequences and import via translocation contact sites was introduced. In terms of the endosymbiont theory the matrix space is equivalent to the prokaryotic cytosol. Translocation into the matrix would therefore bring precursors back on to their 'ancestral' assembly pathway. In the case of Fe/S-protein, this leads to translocation across the pertinent membrane, i.e. the inner cristae membrane which is equivalent to the prokaryotic photosynthetic membrane. The same argument applies if the photosynthetic membrane is contiguous with the plasma membrane (rather than discrete as shown in Fig. 1*a*) because then the Fe/S protein would need to traverse the latter membrane to reach its final destination from the cytosol. We suggest that this 'conservative sorting' hypothesis also applie to a number of other mitochondrial proteins.

Import of ADP/ATP-carrier from the cytoplasm into the inner mitochondrial membrane can be separated into at least four different steps (Fig. 2). After binding to receptor sites on the outer surface of mitochondria, precursor reaches an intermediate location before entrance into the inner membrane. Energy-dependent interaction of the precursor with the inner membrane is then mediated by translocation contact sites. Finally, complete transport into the inner membrane occurs without further requirement of a membrane potential.

In contrast to the different components of the bc_1-complex, proteins like the ADP/ATP-carrier (and the uncoupling protein of brown fat mitochondria) have no known equivalent in prokaryotes (Klingenberg, 1985). These proteins might have been introduced into the organelles later in evolution, i.e. after endocytosis of the endosymbiont.

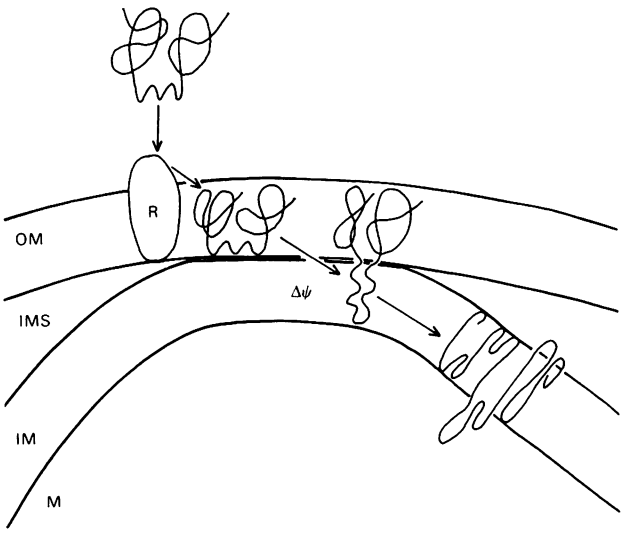

Fig. 2. *Hypothetical pathway for the translocation of ADP/ATP-carrier into mitochondria*

Abbreviations are as in Fig. 1.

It remains to be determined whether absence of a cleavable presequence is related to this possibly different evolutionary origin. On the other hand, the ADP/ATP-carrier also appears to be imported via translocation contact sites. This emphasizes the general importance of these structures for mitochondrial protein import.

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Received 27 August 1986

Molecular mechanism of ATP synthesis in oxidative phosphorylation

HARVEY S. PENEFSKY

Department of Biochemistry, Public Health Research Institute, 455 First Avenue, New York, NY 10016, U.S.A.

There are currently a number of interesting, and as yet only partly resolved, questions in mitochondrial bioenergetics. Two of these deal with the formation of ATP during oxidative phosphorylation and can be stated as follows. (1) How is ATP formed during oxidative phosphorylation? That is, can one write chemical equations that describe the mechanism of ATP synthesis from ADP and P_i? (2) How is energy, represented by electrochemical proton gradients generated during oxidations in the respiratory chain, utilized to drive ATP formation? Since we know that the mitochondrial ATPase (F_1) catalyses ATP synthesis in oxidative phosphorylation, these questions can be reformulated: (1) What is the mechanism of action of F_1 and (2) how is the energy, store coupled to ATP synthesis by the membrane-bound enzyme? This paper addresses both questions.

Several years ago, I undertook with Charles Grubmeyer (Grubmeyer & Penefsky, 1981*a*,*b*), and Richard Cross and

Abbreviations used: TNP, trinitrophenyl; DCCD, dicyclohexylcarbodi-imide.