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Transport of proteins to the mitochondrial intermembrane space: the 'sorting' domain of the cytochrome c_1 presequence is a stop-transfer sequence specific for the mitochondrial inner membrane

Adolphus P.G.M.van Loon[1] and Gottfried Schatz

Biocenter, University of Basel, CH-4056 Basel, Switzerland

[1]Present address: Department of Biochemistry, Central Research Unit (ZFE), Building 15-6A, Hoffmann-La Roche and Co. AG, Grenzacherstrasse 124, CH-4002 Basel, Switzerland

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The presequence of yeast cytochrome c_1 (an inner membrane protein protruding into the intermembrane space) contains a matrix-targeting domain and an intramitochondrial sorting domain. This presequence transports attached subunit IV of cytochrome *c* oxidase into the intermembrane space (van Loon *et al.* (1987) *EMBO J.*, 6, 2433-2439). In order to determine how this fusion protein reaches the intermembrane space, we studied the kinetics of its import into isolated mitochondria or mitoplasts and its accumulation in the various submitochondrial compartments. The imported, uncleaved fusion precursor and a cleavage intermediate were bound to the inner membrane and were always exposed to the intermembrane space; they were never found at the matrix side of the inner membrane. In contrast, analogous import experiments with the authentic subunit IV precursor, or the precursor of the iron- sulphur protein of the cytochrome bc_1 complex (also an inner membrane protein exposed to the intermembrane space), readily showed that these precursors were initially transported across both mitochondrial membranes. We conclude that the intramitochondrial sorting domain within the cytochrome c_1 presequence prevents transport of attached proteins across the inner, but not the outer membrane: it is a stop-transfer sequence for the inner membrane. Since the presequence of the iron-sulphur protein lacks such a 'stop-transfer' domain, it acts by a different mechanism.

Key words: mitochondrial intermembrane space/pre-cytochrome c_1/iron-sulphur protein/subunit IV of cytochrome *c* oxidase/intramitochondrial sorting/stop-transfer sequence

Introduction

The presequence of cytochrome c_1, an inner membrane protein protruding into the intermembrane space (Li *et al.*, 1981a), contains all the information for transporting attached proteins (e.g. mouse dihydrofolate reductase or mature subunit IV of cytochrome *c* oxidase) into the mitochondrial intermembrane space (van Loon *et al.*, 1986, 1987). This presequence contains two functionally distinct domains: a potential matrix-targeting domain (its first quarter) and an intramitochondrial sorting domain (its second half). The matrix-targeting domain, by itself, transports attached proteins into the mitochondrial matrix. The intramitochondrial sorting domain, which contains a stretch of 19 uncharged residues followed by two acidic amino acids (Sadler *et al.*, 1984), by itself appears to be inactive in intracellular sorting, but is required for reaching the intermembrane space (van Loon *et al.*, 1986, 1987). We have previously suggested that the sorting domain of the cytochrome c_1 presequence functions as a stop-transfer sequence that specifically prevents transport of an attached protein across the inner membrane (Daum *et al.*, 1982a; Hurt and van Loon, 1986). A similar model has been proposed for the presequence of cytochrome *c* peroxidase, another heme protein of the yeast mitochondrial intermembrane space (Kaput *et al.*, 1982).

A different import pathway has been suggested for the iron-sulphur protein of the cytochrome bc_1 complex of *Neurospora crassa* (Hartl *et al.*, 1986). This protein, like cytochrome c_1, is an inner membrane protein protruding into the intermembrane space (Bell *et al.*, 1979; von Jagow and Sebald, 1980; Li *et al.*, 1981b; Karlsson *et al.*, 1983). Its precursor form is completely transported into the matrix where it is cleaved to an intermediate by the matrix-located protease. This fact led Hartl *et al.* (1986) to suggest that intermembrane space proteins are first transported into the matrix; removal of an N-terminal part of the presequence by the matrix protease would then allow the intermediate to be re-exported across the inner membrane to the intermembrane space.

In order to determine which of these two models applies to the cytochrome c_1 presequence we studied the pathway by which three different precursor proteins are imported into the various compartments of isolated mitochondria or mitoplasts (mitochondria whose outer membranes have been disrupted). The

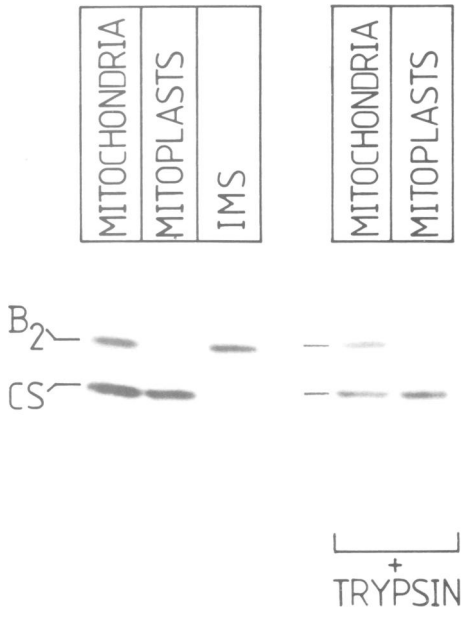

Fig. 1. Characterization of mitochondria and mitoplasts which were used for *in vitro* import experiments. **Left panel:** mitochondria were incubated in 0.06 M sorbitol buffer to break the outer membrane. The resulting mitoplasts were separated from the intermembrane space fraction (IMS) by centrifugation. **Right panel:** mitochondria or equivalent amounts of mitoplasts were used for *in vitro* protein import, treated with trypsin and subjected to SDS-10% polyacrylamide gel electrophoresis and immune blotting with a mixture of antibodies against the intermembrane space marker cytochrome b_2 (B_2) and the matrix marker citrate synthase (CS).

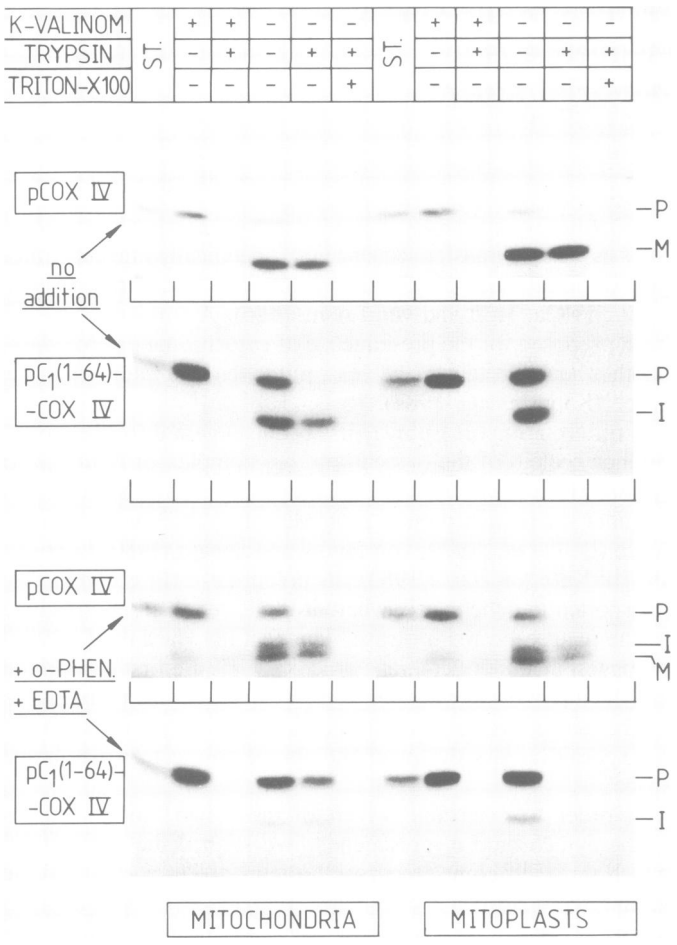

Fig. 2. Import of authentic subunit IV precursor and of the $pC_1(1-64)-$COX IV fusion protein into mitochondria or mitoplasts is energy-dependent and removal of the presequence involves the matrix-located processing protease. The authentic subunit IV precursor (pCOX IV) or the $pC_1(1-64)-$COX IV fusion protein were synthesized *in vitro* in the presence of [^{35}S]methionine and incubated with mitochondria or mitoplasts. After incubation for 30 min at 18°C, the organelles were re-isolated by centrifugation and, where indicated, treated with trypsin (in the absence or presence of the detergent Triton X-100) prior to electrophoretic analysis. In some cases, the organelles were incubated in the presence of valinomycin plus KCl (K-VALINOM.; to de-energize the inner membrane) and/or of *o*-phenantroline plus EDTA (+ o-PHEN. + EDTA; lower two panels; to partially inhibit the matrix-located processing protease). Radiolabelled proteins were visualized by fluorography after electrophoresis on SDS−13.5% polyacrylamide gels. ST, 20% of the amount of the radiolabelled precursor added to each of the other incubations; P, precursor; I, cleavage intermediate; M, mature-sized subunit IV.

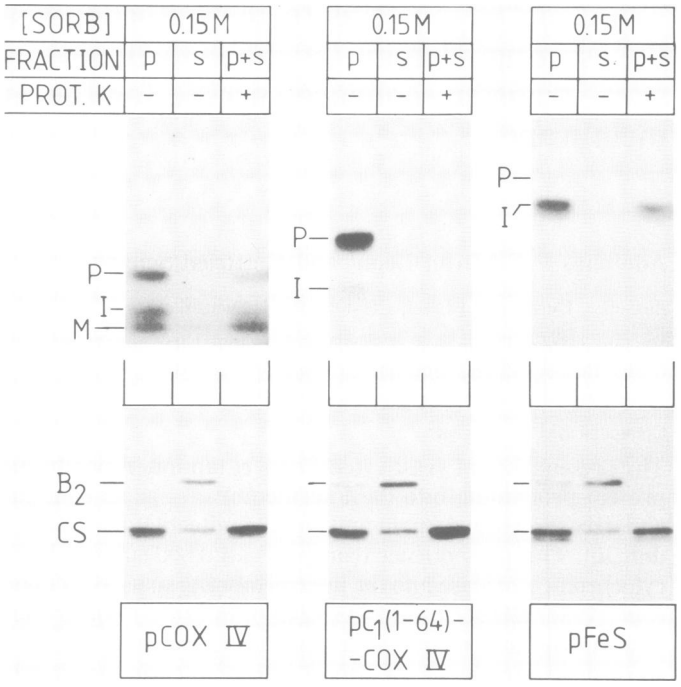

Fig. 3. Upon import into mitochondria whose matrix-located protease had been inhibited, the uncleaved $pC_1(1-64)-$COX IV fusion protein is located between the outer and inner membranes whereas uncleaved or cleaved forms of the subunit IV precursor or the intermediate of iron−sulphur protein precursor are found at the matrix side of the inner membrane. The radiolabelled subunit IV precursor (pCOX IV), the $pC_1(1-64)-$COX IV fusion protein and the iron−sulphur protein precursor (pFeS) were incubated for 15 min at 18°C with energized mitochondria in the presence of *o*-phenantroline and EDTA. Incubation was stopped by addition of a 4-fold excess of ice-cold stop buffer (see Materials and methods) containing 250 μg trypsin/ml to remove all proteins present at the mitochondrial surface. After incubation at 0°C for 15 min, the mitochondria were diluted to 0.15 M sorbitol to break the outer membrane. Each sample was split into two halves. One half was centrifuged to separate mitoplasts (p) from intermembrane space fraction (s) while the other half (p + s) was incubated with proteinase K. Each sample was again split in two halves which were applied to different SDS−polyacrylamide gels (13.5%, **top panel**; 10%, **bottom panel**). Proteins were visualized by fluorography **(top panel)** or by immune blotting **(bottom panel)** with a mixture of antibodies against cytochrome b_2 (B_2) and citrate synthase (CS). P, precursor; I, cleavage intermediate; M, mature-sized subunit IV.

three precursor proteins were (i) a fusion protein containing the cytochrome c_1 presequence fused to mature subunit IV of cytochrome *c* oxidase [$pC_1(1-64)-$COX IV]; (ii) the authentic subunit IV precursor (pCOX IV); and (iii) the authentic precursor of the iron−sulphur protein (pFeS) of the cytochrome bc_1 complex. We readily confirmed the observation by Hartl *et al.* (1986) that the iron−sulphur protein is initially completely transported into the matrix space and also found that the subunit IV precursor follows a closely similar import route. In contrast, mature subunit IV carrying the cytochrome c_1 presequence never completely crossed the inner membrane during its import into the intermembrane space. These results strongly suggest that the intramitochondrial sorting domain of the cytochrome c_1 presequence is a stop−transfer sequence specific for the mitochondrial inner membrane as suggested before (Daum *et al.*, 1982a; Hurt and van Loon, 1986). Thus, the precursors of cytochrome c_1 and the iron−sulphur protein use different pathways to reach the intermembrane space.

Results

Authentic subunit IV precursor and subunit IV attached to the cytochrome c_1 presequence are both cleaved by isolated mitochondria or mitoplasts but only authentic subunit IV precursor is completely transported into the matrix

In order to define the routes of these two precursor proteins during their import into yeast mitochondria, it was essential to prepare intact mitochondria and mitoplasts free of intact mitochondria. Figure 1 shows that this was achieved. In intact mitochondria, cytochrome b_2 (an intermembrane space enzyme) and citrate synthase (a matrix enzyme) were resistant to externally added protease; in mitoplasts, cytochrome b_2 was essentially undetectable, but citrate synthase was still protease inaccessible. These mitochondria and mitoplasts (or preparations of comparable quality) were used for the protein import experiments described below. All import experiments described in this paper were done

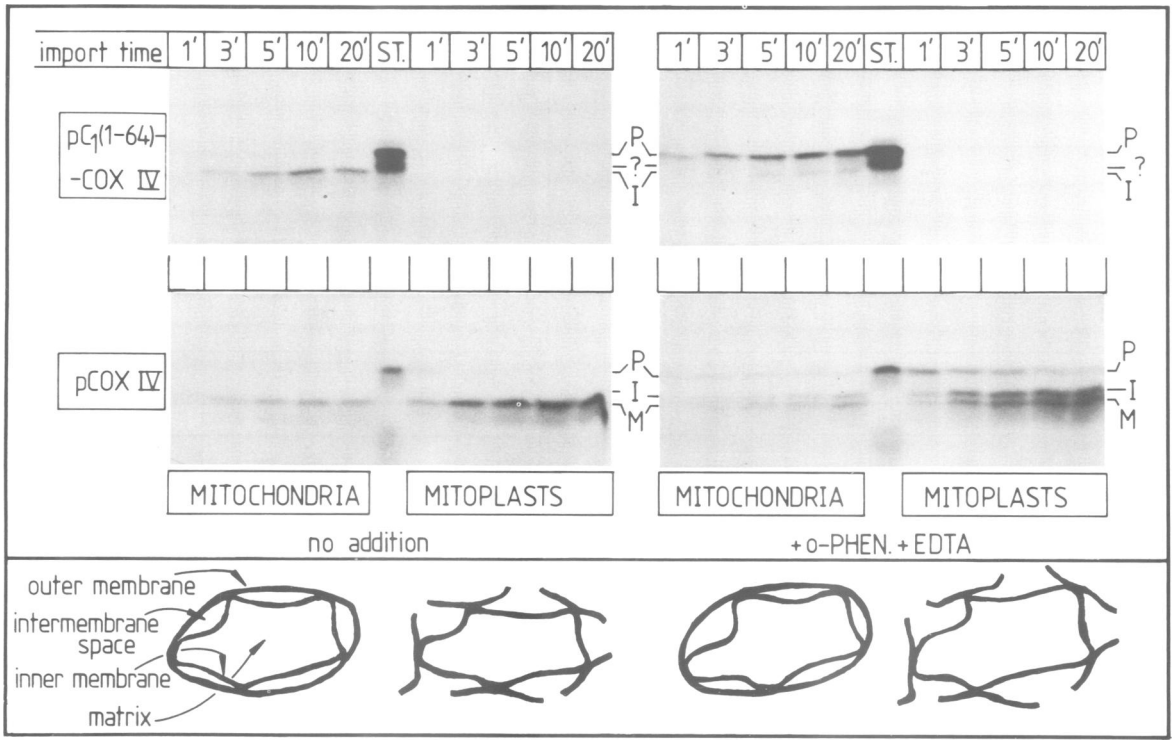

Fig. 4. Fluorograms showing that the $pC_1(1-64)-$COX IV fusion protein and its cleavage intermediate are transported across the mitochondria outer membrane but not across the inner membrane, whereas the authentic subunit IV precursor is transported across both membranes. Radiolabelled subunit IV precursor (pCOX IV) and the $pC_1(1-64)-$COX IV precursor were incubated with energized mitochondria or mitoplasts, either in the absence (no addition) or in the presence of *o*-phenantroline plus EDTA (+ o-PHEN. + EDTA). At the indicated times, samples were diluted into ice-cold, trypsin-containing stop buffer (see Materials and methods). After incubation at 0°C for 10–15 min the organelles were isolated by centrifugation and radiolabelled proteins were visualized by electrophoresis on SDS–13.5% polyacrylamide gels and fluorography. ST, 20% of the amount of the radiolabelled precursor added to each of the other incubations; P, precursor; I, cleavage intermediate; M, mature-sized subunit IV; ?, an unknown radiolabelled contaminant present in this particular preparation of $pC_1(1-64)-$COX IV fusion protein. This contaminant does not bind to mitochondria and its electrophoretic mobility is distinctly lower than that of the cleavage intermediate.

at 18°C in order to slow down import and facilitate detection of import intermediates.

When the authentic subunit IV precursor (pCOX IV) was imported into energized mitochondria (Figure 2, top panel), it was converted to mature-sized subunit IV. The mature subunit (M) was inaccessible to externally added protease unless mitochondrial membranes were disrupted by Triton X-100 (cf. also Hurt *et al.*, 1984). The same result was obtained with energized mitoplasts (Figure 2, top panel). Import into mitoplasts was even more efficient than into intact mitochondria (see also below); most of the added precursor molecules were imported and cleaved. No cleavage or import occurred if mitochondria or mitoplasts were de-energized by K^+ plus valinomycin. When the matrix-located processing protease was partly inhibited by *o*-phenantroline and EDTA (Boehni *et al.*, 1983), some of the subunit IV precursor molecules accumulated in the matrix in uncleaved form (P) or as a cleavage intermediate (I): they were protease-resistant after import into mitochondria and into mitoplasts as well (Figure 2, 3rd panel from top). The existence of two cleavage sites in the subunit IV presequence had been noted earlier (Hurt *et al.*, 1985). These control experiments confirmed that the mitochondria and mitoplasts used here could efficiently import the subunit IV precursor into their matrix space, that import was energy dependent, that it could still occur if the matrix protease had been partly inhibited, and that the inner membranes of the mitoplasts used here could protect matrix-located proteins from externally added trypsin.

Subunit IV carrying the cytochrome c_1 presequence [i.e. the $pC_1(1-64)-$COX IV fusion protein] behaved differently (Figure 2, 2nd panel from top): it was efficiently imported by intact energized mitochondria, but was only converted to a cleavage intermediate (I). [Cleavage to mature subunit IV and release of mature subunit IV into the intermembrane space only occurred at a higher temperature (van Loon *et al.*, 1987).] The cleavage intermediate accumulating at 18°C was inaccessible to externally added protease; it was not formed by de-energized mitochondria. When the matrix protease was partially inhibited (Figure 2, bottom panel), then the fusion precursor was imported but only very inefficiently cleaved: a major fraction of the mitochondrially associated precursor molecules was resistant to externally added protease. They were transported across the outer membrane.

The cleavage intermediate (I) was also seen upon incubation with energized mitoplasts (Figure 2, 2nd panel from top), but it was now fully accessible to externally added trypsin: it was not transported across the inner membrane. If the matrix protease was inhibited (Figure 2, bottom panel), most of the radiolabelled fusion protein remained uncleaved (P); it was fully accessible to externally added protease: the imported uncleaved fusion protein, too, was not transported across the inner membrane. This indicates that subunit IV carrying its own presequence is transported across both mitochondrial membranes whereas subunit IV carrying the cytochrome c_1 presequence is only transported across the outer membrane, but not the inner membrane.

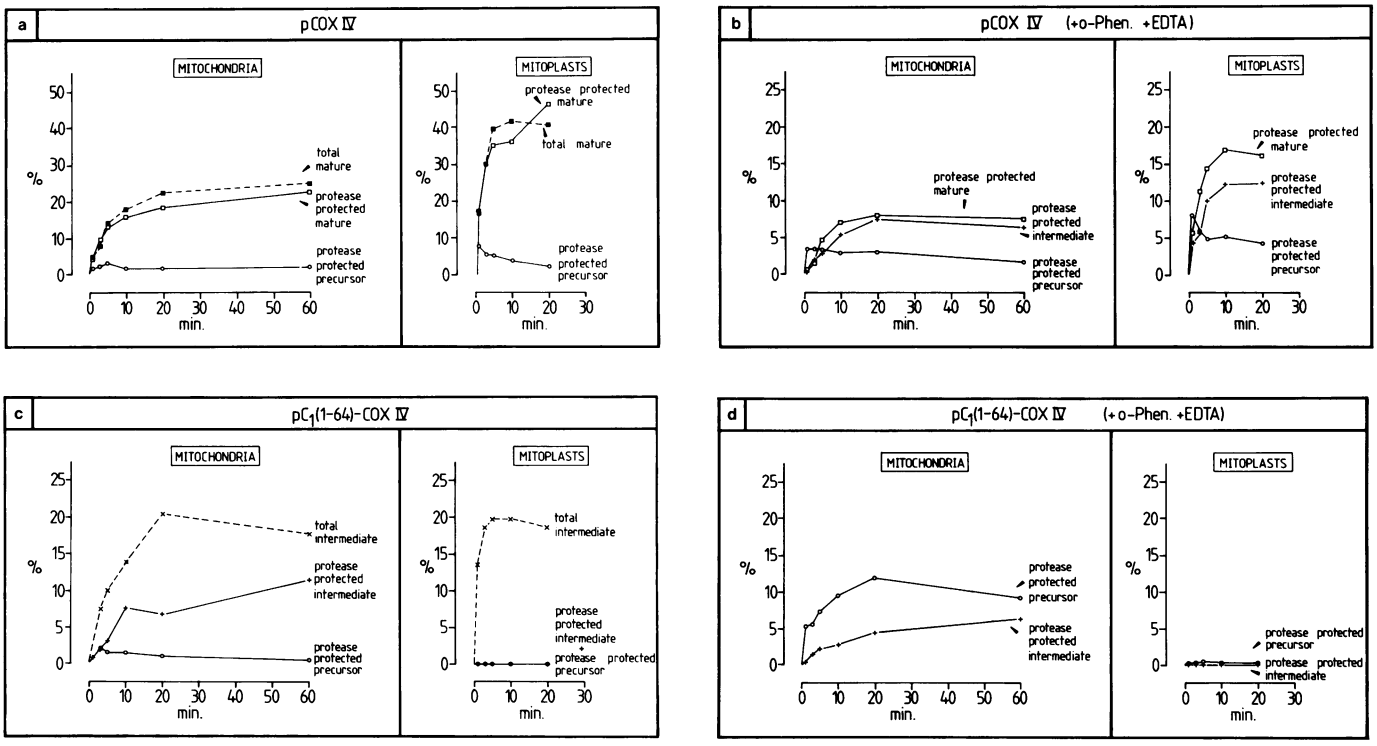

Fig. 5. Quantitation of fluorograms showing that the $pC_1(1-64)-COX$ IV fusion protein and its cleavage intermediate are transported across the mitochondrial outer membrane but not across the inner membrane, whereas the authentic subunit IV precursor is transported across both membranes. Fluorograms, some of which are shown in Figure 4, were quantified as described by Suissa (1983). The 'total intermediate' of the $pC_1(1-64)-COX$ IV precursor was quantitated using fluorograms of gels that contained samples not treated with trypsin after protein import (see Results). The 'total precursor' was not quantitated since a large fraction of the precursor molecules was bound to the mitochondrial surface. **Panel B:** mature-sized subunit IV and the cleavage intermediate were completely protected against externally added protease; the total amount of intermediate and of the mature subunit are not shown, however. %, percentage of the total amount of radiolabelled precursor added to each of the incubations.

Upon import into mitochondria whose matrix-located protease is inhibited, subunit IV carrying the cytochrome c_1 presequence is located between the outer and inner membranes

The authentic subunit IV precursor (pCOX IV), the $pC_1(1-64)-COX$ IV fusion protein and the authentic yeast iron–sulphur protein (pFeS) were imported into mitochondria in the presence of *o*-phenantroline and EDTA at 18°C for 15 min (Figure 3). In this condition, import increases linearly with time (see below). Import and precursor cleavage were stopped by dilution of the samples with ice-cold stop buffer. This buffer contained several inhibitors that de-energize the inner membrane and inhibit ATP synthesis and the matrix-located processing protease (see Materials and methods); it also contained trypsin to digest all proteins present on the mitochondrial surface. After incubation at 0°C mitochondria were re-isolated and converted to mitoplasts. Each sample was divided into two aliquots. One aliquot was centrifuged to separate mitoplasts (pellet, p) from the solubilized intermembrane space contents (supernatant, s); the other aliquot (p + s) was treated with proteinase K. Effectiveness of mitochondrial subfractionation was ascertained by assaying the distribution and protease accessibility of the intermembrane space enzyme cytochrome b_2 and of the soluble matrix enzyme citrate synthase. Cytochrome b_2 was almost exclusively found in the supernatant fractions and was fully protease sensitive. In contrast, citrate synthase was retained in the mitoplast pellet and was resistant to externally added protease (Figure 3, lower panel).

On assaying the submitochondrial distribution of the imported radiolabelled precursor, the uncleaved $pC_1(1-64)-COX$ IV fusion protein (P) was located between the outer and inner membranes: it was membrane-bound, but protease-sensitive in mitoplasts (Figure 3, top panel). In contrast, uncleaved (P), partly cleaved (I) and completely cleaved subunit IV precursor (M) and the partly cleaved iron–sulphur precursor protein (I) were found on the matrix side of the inner membrane: they were all resistant to proteinase K digestion of mitoplasts (Figure 3, top panel). If Triton X-100 was added prior to protease digestion, all proteins were completely digested (not shown). Thus, the imported, uncleaved $pC_1(1-64)-COX$ IV fusion protein was found between the outer and inner membranes even under conditions in which import was linear with time and in which other precursors and partly cleaved precursor proteins were readily found on the matrix side of the inner membrane.

The cytochrome c_1 presequence transports subunit IV across the outer membrane in mitochondria, but not across the inner membrane in mitoplasts: time course experiments

To exclude that the $pC_1(1-64)-COX$ IV fusion protein was initially transported into the matrix and then quickly exported to the intermembrane space, we imported the $pC_1(1-64)-COX$ IV fusion protein into isolated mitochondria or mitoplasts and determined the sensitivity of the imported protein to externally added protease at various times after import. For comparison, the same time course experiments were also done in parallel for the authentic subunit IV precursor. Both proteins were synthesized *in vitro* and incubated with energized mitochondria or mitoplasts. At the indicated times, two samples were withdrawn and directly mixed with ice-cold stop buffer (cf. above). The stop buffer for one of the two samples lacked trypsin. Both samples were incubated at 0°C; mitochondria or mitoplasts were then isolated

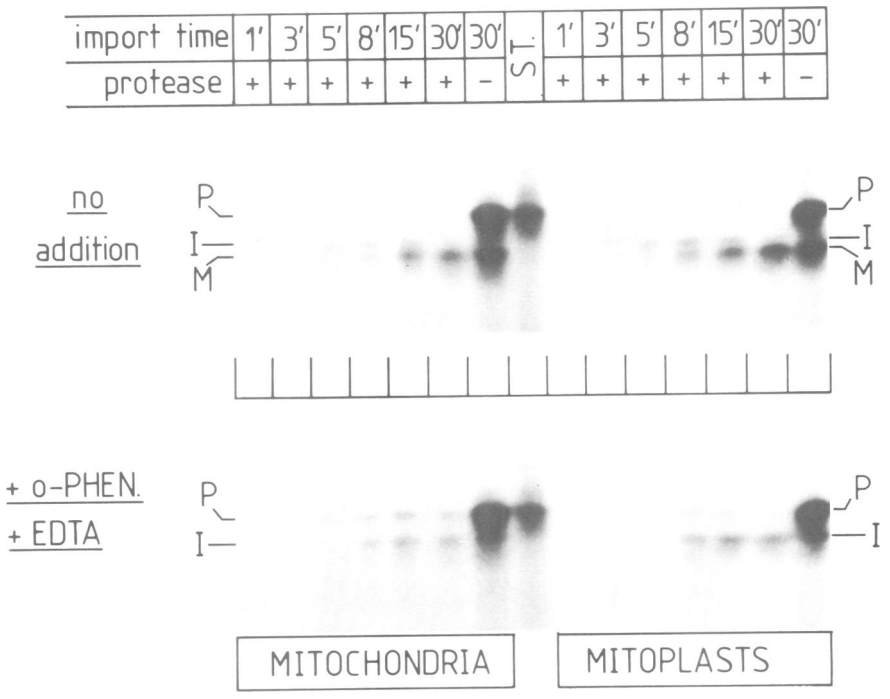

Fig. 6. The precursor of the iron-sulphur protein of the cytochrome bc_1 complex is completely transported across the inner membrane. The radiolabelled precursor of the iron-sulphur protein was incubated with energized mitochondria or mitoplasts, either in the absence (no addition) or in the presence of *o*-phenantroline plus EDTA (+ o-PHEN. + EDTA). At the indicated times, two samples were taken and diluted with ice-cold stop buffer (see Materials and methods). Trypsin was omitted from the stop buffer used for one of each two samples. After incubation at 0°C for 10-15 min, the organelles were isolated by centrifugation and radiolabelled proteins were visualized by electrophoresis on SDS-13.5% polyacrylamide gels and fluorography. ST, 20% of the radiolabelled precursor added to each of the other incubations; P, precursor; I, cleavage intermediate; M, mature-sized iron-sulphur protein.

by centrifugation and analysed for radiolabelled proteins by SDS-polyacrylamide gel electrophoresis and fluorography (Figure 4 shows some of the gels, while the quantitations of the results are shown in Figure 5).

Even after the shortest import times, the partly cleaved pC_1-(1-64)-COX IV fusion protein (I) was (in part) inaccessible to external protease in mitochondria, but fully accessible to external protease in mitoplasts (Figure 4, top left panel and Figure 5C). If the matrix-localized protease in the mitochondria or mitoplasts was inhibited by *o*-phenantroline and EDTA, the fusion protein (P) was still imported, but not cleaved. Again, the imported precursor molecules were only found between the two membranes in mitochondria (cf. Figure 3) and at the outer surface of the inner membrane in mitoplasts (Figure 4, top right panel and Figure 5D): the precursor never became protease resistant in mitoplasts. Thus, at no time were precursor molecules found on the matrix side of the inner membrane.

In contrast, the subunit IV precursor (P) and its two cleavage products (I and M) were transported across the inner membrane, both in mitochondria and mitoplasts (Figure 4, bottom panels and Figure 5A, B). The partly cleaved form (I) appears to be a *bona fide* intermediate: if import occurred at an even lower temperature (10°C), we could clearly see initial conversion of precursor (P) to intermediate (I) and later conversion of intermediate (I) to mature subunit IV (M) (not shown).

With both precursors import into mitoplasts was faster than into intact mitochondria. Transport across the outer membrane may, thus, have been rate-limiting under these conditions.

The precursor of the iron-sulphur protein is transported across the inner membrane

The precursor to the iron-sulphur protein of the yeast cytochrome bc_1 complex was synthesized *in vitro* and imported into isolated mitochondria or mitoplasts as described above. Samples were taken at the indicated times and diluted with ice-cold stop buffer, with or without trypsin, as indicated. The organelles were re-isolated after incubation and analysed for the presence of radiolabelled proteins. Figure 6 shows that precursor (P), cleavage intermediate (I) and the mature iron-sulphur protein (M) became inaccessible to externally added protease upon import into both mitochondria and mitoplasts: they were transported across the inner membrane. A low but significant fraction of precursor and most of the cleavage intermediate was protease protected in mitochondria and mitoplasts. Most of the precursor molecules were present, however, at the outer surface of the outer membrane: they were protease sensitive even in intact mitochondria. This indicated that they only bound to mitochondria but were not imported. This binding may be aspecific. In mitochondria the total amounts of protease-protected precursor plus intermediate (as fraction of the total amount of precursor added to mitochondria) were similar for the pC_1(1-64)-COX IV and the iron-sulphur protein (compare Figure 4, top right panel, with Figure 5, lower panel). Thus, the precursor of the iron-sulphur protein behaved in the same way as the authentic subunit IV precursor: both were transported into the matrix and differed clearly from the pC_1(1-64)-COX IV fusion protein which was only found in the intermembrane space. Even though the iron- sulphur protein precursor protein and the pC_1(1-64)-COX IV fusion protein both reach the intermembrane space, they do so by at least partly different pathways.

Discussion

The problem

Two different pathways have been proposed by which proteins are transported from the cytosol to the mitochondrial intermem-

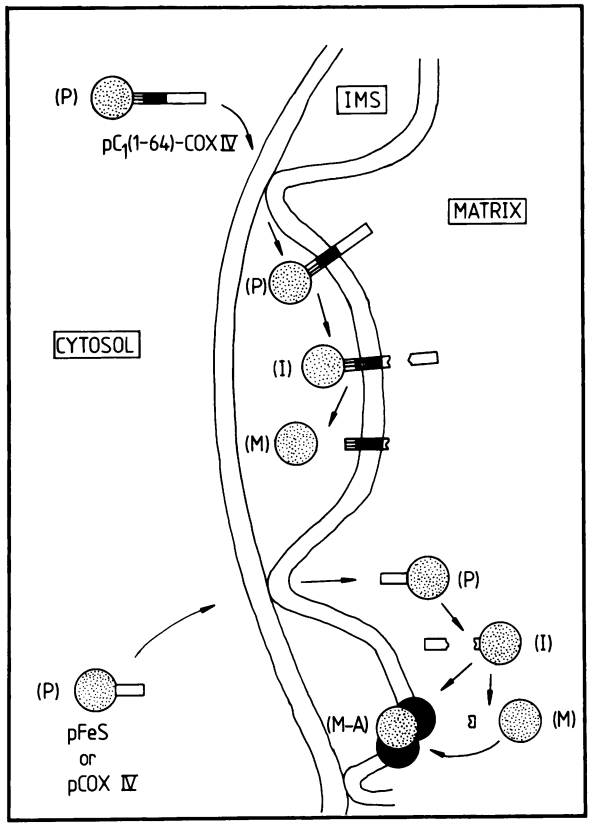

Fig. 7. Two different ways to reach the mitochondrial intermembrane space. This figure summarizes the data presented in this paper and by Hartl *et al.* (1986). It is unclear whether the pFeS and pCOX IV precursors in the matrix are associated with the inner membrane and whether complex asembly occurs before or after formation of the mature proteins. Open box, matrix-targeting-like sequences containing basic but no acidic residues and lacking long stretches of uncharged amino acids. Filled-in box, stretch of 19 uncharged residues in the cytochrome c_1 presequence (amino acids 36–54). Box with horizontal lines, carboxy-terminal part of the cytochrome c_1 presequence which contains two acidic residues. Stippled circles, mature subunit IV or mature iron–sulphur protein. Filled-in circles, subunits of assembled respiratory chain complexes. IMS, intermembrane space; P, precursor; I, cleavage intermediate; M, mature-sized protein; M–A, mature-sized protein assembled into the respiratory chain complex.

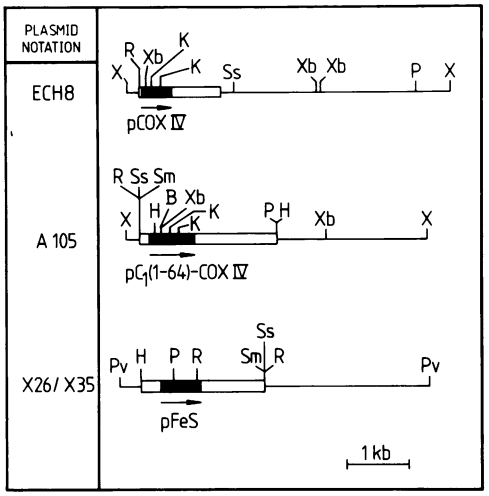

Fig. 8. Plasmids for *in vitro* transcription of genes. The vectors (thin lines) are pDS5-2 (in plasmid ECH8), pDS5 (in plasmid A105) and pGEM2 (in plasmids X26 and X35). Expression of the genes is under control of the T5 promoter in pDS5-2 and pDS5 (Stueber *et al.*, 1984) or under control of the T7 promoter in pGEM2. Filled-in boxes, (fusion) genes; open boxes, non-coding sequences not originating from the vectors. The arrows indicate the positions of the genes and the directions of transcription. The abbreviations used for restriction enzymes are: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; Sm, *Sma*I; Ss, *Sst*I; X, *Xho*I; Xb, *Xba*I.

brane space. In this study we have asked whether these two pathways operate side-by-side for different sets of proteins, or whether one of the previous suggestions was in error. In that case, there might only be a single pathway which is followed by all proteins transported into the intermembrane space.

The first pathway was formulated for cytochrome c_1 and two other heme proteins (Ohashi *et al.*, 1982; Daum *et al.*, 1982a; Kaput *et al.*, 1982; van Loon *et al.*, 1986; Hurt and van Loon, 1986). This model suggested that only part of the presequence is transported across the inner membrane and then cleaved off by the matrix protease, while the bulk of the protein never crosses the inner membrane. The model was based on the unexpected observation that import of cytochrome c_1 to its normal location at the outer face of the inner membrane requires at least partial translocation across the inner membrane. Thus, import depends on an energized inner membrane and is accompanied by proteolytic removal of the presequence in two successive steps, the first of them occurring in the matrix space.

Subsequent experiments showed that the 61-residue presequence of cytochrome c_1 contains all the necessary information for transport into the intermembrane space: when the presequence was fused to the cytosolic enzyme mouse dihydrofolate reductase, the resulting fusion protein was imported into mitochondria and cleaved in two distinct steps, the first of them occurring in the matrix. As a result, presequence-free dihydrofolate reductase was deposited in the soluble intermembrane space (van Loon *et al.*, 1986). DNA sequence analysis of the cytochrome c_1 gene (Sadler *et al.*, 1984) revealed the presence of two structurally distinct domains in the presequence. These domains were also functionally distinct: a positively charged N-terminal domain (16 residues or less) could, by itself, transport attached proteins to the matrix; and an uninterrupted stretch of 19 uncharged residues (residues 36–54) followed by a short region with two negatively charged residues modulated the 'matrix-targeting' signal so that attached proteins were deposited in the intermembrane space (van Loon *et al.*, 1986, 1987). We suggested that this 'sorting domain' was, in fact, a stop–transfer sequence which prevented passage of attached proteins across the inner membrane.

The second pathway was recently proposed for the iron–sulphur protein of the *N. crassa* cytochrome bc_1 complex (Hartl *et al.*, 1986). This protein, like cytochrome c_1, is assembled into the transmembraneous cytochrome bc_1 complex such that it is partly exposed towards the intermembrane space (Bell *et al.*, 1979; von Jagow and Sebald, 1980; Li *et al.*, 1981b; Karlsson *et al.*, 1983). Again like cytochrome c_1, import of the precursor into mitochondria requires an energized inner membrane and is accompanied by two successive cleavages, the first of them by the matrix-localized processing protease. Unlike cytochrome c_1, however, imported precursor and intermediate cleavage product can be detected within the matrix space before the mature protein is generated. This led Hartl *et al.* (1986) to propose that the precursor was initially transported to the matrix and that removal of an N-terminal domain by the matrix protease would allow the cleavage intermediate to be re-exported across the inner membrane to the intermembrane space. According to this model, the C-terminal part of the presequence is not a 'stop–transfer' sequence specific for the inner membrane, but may be an 'export' signal resembling the signal sequence of an exported bacterial protein. Hartl *et al.* suggested that this pathway might be followed

by many other proteins which are imported into the intermembrane space.

These pathways differ by two predictions which can be experimentally tested. According to the first pathway, the precursors should never completely enter the matrix space and inhibition of the first cleavage step should not prevent appearance of the protein in the intermembrane space. According to the second pathway, the precursors should completely enter the matrix space and be unable to leave the matrix if the first cleavage step is blocked.

Here we show that the cytochrome c_1 presequence imports attached proteins by the first pathway whereas two other proteins are imported by the second pathway.

Experimental approach

For our experiments we used cytochrome *c* oxidase subunit IV linked either to its own presequence (i.e. the authentic precursor) or to the cytochrome c_1 presequence [i.e. the $pC_1(1-64)-$COX IV fusion protein]. Both proteins differ only in their presequences and, as a result, in their final intramitochondrial location after import into mitochondria (van Loon *et al.*, 1987; and this paper). We decided to test the function of the cytochrome c_1 presequence with the aid of this fusion protein rather than with the authentic cytochrome c_1 precursor since import of the authentic precursor might be complicated by assembly of mature cytochrome c_1 into the cytochrome bc_1 complex. This assembly might be partly mediated by the hydrophobic C-terminal domain of the mature cytochrome c_1 (Wakabayashi *et al.*, 1980). In contrast, subunit IV attached to the cytochrome c_1 presequence is deposited in the intermembrane space without being assembled into cytochrome *c* oxidase (van Loon *et al.*, 1987), yet exhibits the same import characteristics and cleavage intermediates as the authentic cytochrome c_1 precursor. This fusion protein is, thus, ideally suited to analyse the function of the cytochrome c_1 presequence without complications of topogenic signals in the 'passenger' protein.

In order to test whether a protein had completely crossed the inner membrane, we imported the precursor either into mitochondria or into mitoplasts (mitochondria whose outer membrane was broken) and checked whether the imported protein was accessible to externally added protease. Import into mitoplasts was preferred above subfractionation of mitochondria after *in vitro* import since mitochondria become quite fragile upon incubation under import conditions; this usually causes artefactual release of variable amounts of matrix together with the intermembrane space fraction.

Here we show that mitochondria and mitoplasts have very similar import characteristics. (i) Import of precursors into mitochondria and into mitoplasts and precursor cleavage was dependent on a potential across the inner membrane. (ii) Cleavage could be inhibited by *o*-phenantroline and EDTA, indicating that the matrix-located processing protease is involved (Boehni *et al.*, 1983). (iii) All proteins which were being transported into mitochondria or mitoplasts (including imported precursor) were alkali soluble. In mitochondria, all of the imported, protease-protected $pC_1(1-64)-$COX IV precursor molecules were alkali soluble. In contrast, precursors bound to the outer membrane were alkali insoluble. In mitoplasts, a major fraction, but not all, of the precursor molecules were alkali soluble (not shown). This strongly suggests that the precursor in mitoplasts is also being transported in a similar way to the internal precursor in mitochondria. (iv) Proteins that became protease resistant upon import into both mitochondria and mitoplasts were found, upon subfractionation of mitochondria, at the matrix side of the inner membrane. (v) Proteins that became protease resistant during import into mitochondria but not during import into mitoplasts were found, upon subfractionation of mitochondria, to be exposed to the mitochondrial intermembrane space. Mitochondria and mitoplasts only differed in the rate with which they imported proteins: mitoplasts imported all three precursors more rapidly and imported a larger percentage of the authentic subunit IV precursor. Comparison of the protease sensitivity of proteins after import into mitochondria and into mitoplasts can, thus, be used to determine whether a protein is completed transported across the mitochondrial inner membrane.

The results

Our experiments on the import of the authentic subunit IV precursor and of the precursor to the iron-sulphur protein readily confirmed the results reported by Hartl *et al.* (1986): the precursors and the cleavage intermediates could be detected inside the inner membrane. However, subunit IV carrying the cytochrome c_1 presequence behaved differently: it was never found inside the inner membrane, even in careful kinetic analyses. It might, of course, be argued that this is a negative result which merely reflects the fact that the rate of import into the matrix is much slower than the rate at which the cleavage intermediate is re-exported. This explanation is, however, unlikely. (i) This faster re-export of the cleavage intermediate would have to be specific for the $pC_1(1-64)-$COX IV precursor since the precursor of the iron-sulphur protein was readily found on the matrix side of the inner membrane. (ii) This explanation cannot account for the fact that the uncleaved fusion protein was imported into the intermembrane space even when its first cleavage in the matrix was inhibited: the protein was protease inaccessible in mitochondria, but not in mitoplasts. This result is incompatible with the second pathway which proposes that only the cleaved intermediate is competent for re-export from the matrix. If this were so, the uncleaved fusion protein should have been trapped in the matrix space. In addition, the $pC_1(1-64)-$COX IV protein did not complement the growth deficiency in a COX IV mutant (van Loon *et al.*, 1987). This observation also supports the idea that the $pC_1(1-64)-$COX IV protein is never transported completely into the matrix: thus far, all COX IV fusion precursors, that contain mature subunit IV and enter the matrix, do complement the COX IV mutant.

Conclusion

We propose that cytoplasmically synthesized proteins can reach the mitochondrial intermembrane space by at least two different pathways (Figure 7). One pathway is selected by the cytochrome c_1 presequence; it involves a stop-transfer sequence specific for the mitochondrial inner membrane. The other pathway is selected by the presequence of the iron-sulphur protein of the cytochrome bc_1 complex and possibly also by the authentic presequence of cytochrome *c* oxidase subunit IV. Both presequences also have very similar characteristics (Maarse *et al.*, 1984; Hartl *et al.*, 1986; Beckman *et al.*, 1987). This second pathway involves a signal for re-export from the matrix. Whether this re-export is mediated by the C-terminal part of the presequence, or by assembly of the mature protein into a transmembrane complex, remains open. Experiments are under way to distinguish between these possibilities.

Materials and methods

Plasmids

The gene for the iron-sulphur protein of the yeast cytochrome bc_1 complex was excised from plasmid YEp351-RIP1 (Beckman *et al.*, 1987) as a *Hin*dIII-*Sma*I

fragment and cloned into the *in vitro* transcription/translation vector pGEM2 (Promega, Inc.). The resulting plasmids are X26 and X35. Expression of the gene was under control of the T7 promoter. The authentic subunit IV gene [in plasmid pECH8, which is the same plasmid as pDS5/2-1 COX IV (Hurt *et al.*, 1984)] and the $pC_1(1-64)-$COX IV fusion gene (in plasmid A105, van Loon *et al.*, 1987) were expressed under control of the T5 promoter. Restriction maps of all plasmids are shown in Figure 8.

In vitro protein synthesis

Radiolabelled proteins were synthesized by coupled transcription/translation. Genes under control of the T5 promoter were expressed as described (Stueber *et al.*, 1984; Hurt *et al.*, 1984). The gene for the iron–sulphur protein was transcribed using 10–20 U of T7 polymerase (Bethesda Research Laboratories) and 1 μg of linearized plasmid per 50 μl incubation for 2 h at 37°C. The incubation contained 40 mM Tris–HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine·$(HCl)_3$, 25 mM NaCl, 5 mM dithiothreitol and 400 μM each of ATP, GTP, CTP and UTP. Transcription was stopped by addition of EDTA to 20 mM, the mixture was extracted with phenol, RNA was precipitated with ethanol, resuspended in 50 μl sterile water and stored at −20°C. It was translated as described by Hurt *et al.* (1984) using 15 μl RNA/100 μl translation mixture.

Mitochondrial import experiments

Mitochondria for *in vitro* import experiments were prepared (Gasser *et al.*, 1982) from the *Saccharomyces cerevisiae* strain D273-10B (*alpha*) (ATCC 25675). Mitochondrial import experiments were done essentially as described by Gasser *et al.* (1982). Incubations were at 18°C in order to slow down the rate of import. Incubation mixtures contained salt, buffer, nucleotides and an ATP-regeneration system (Gasser *et al.*, 1982) as well as 1–2 mg mitochondrial or mitoplast proteins/ml and ~10% (v/v) reticulocyte lysate. Where indicated, mitochondria were de-energized with valinomycin (1–2 μg/ml) before addition of the reticulocyte lysate.

The import mix, mitochondria or mitoplasts, and reticulocyte lysate containing the radiolabelled fusion protein were pre-incubated at 18°C; at the indicated times, samples were withdrawn and mixed with a 4-fold excess of ice-cold buffer containing 0.6 M sorbitol, 20 mM Tris–HCl (pH 7.4), 20 μM carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP; an uncoupler of oxidation phosphorylation), 4 μM oligomycin, 1–2 μg valinomycin/ml, 100 mM KCl, 0.5 mM *o*-phenantroline and 10 mM EDTA (stop buffer) and (where indicated) 250–300 μg tryspin (bovine, TPCK-treated, 3.5 U/μg, Merck)/ml. The stop buffer also contained 2–4% ethanol as a result of adding inhibitors from stock solutions in ethanol. After dilution, the samples contained ~0.2 mg mitochondria or mitoplasts/ml. They were incubated in ice-water for 10–15 min and soybean trypsin inhibitor was then added to 0.5 mg/ml, even to samples that had not received trypsin. Mitochondria or mitoplasts were re-collected by centrifugation, resuspended in stop buffer containing 100 μg trypsin inhibitor/ml and processed for gel electrophoresis. For partial inhibition of the matrix-located processing protease, *o*-phenantroline and EDTA (final concentrations of 0.1 and 10 mM respectively) were added to mitochondria or mitoplasts in the import mix ~3 min before the reticulocyte lysate containing radiolabelled proteins. Digestions with proteinase K (from *Tritirachium album*, 9.3 DMC-U/mg, Serva) (1 DMC-U hydrolyses 1 μmol dimethylcasein/min at 25°C, pH 7.0) were done at 0°C for 30 min in 0.6 M sorbitol, 20 mM Tris–HCl (pH 7.4) at the following final concentrations: mitochondria or mitoplasts, 0.5–2 mg/ml; proteinase K, 100 μg/ml. Digestion was stopped by addition of phenylmethylsulphonyl fluoride to 1 mM. Where indicated protease treatment was done in the presence of 0.5% Triton X-100.

Miscellaneous

Mitoplasts were prepared essentially as described by Daum *et al.* (1982b). Isolated mitochondria (~10 mg/ml in 0.6 M sorbitol and 20 mM Tris–HCl, pH 7.4) were diluted 10-fold with ice-cold 20 mM Tris–HCl (pH 7.4). After incubation on ice for 15–20 min and occasional mixing using a Dounce homogenizer, the resulting mitoplasts were collected by centrifugation and resuspended to the concentration of the original mitochondrial suspension in 0.6 M sorbitol, 20 mM Tris–HCl (pH 7.4).

Published procedures were used for immune blotting (Haid and Suissa, 1983), for alkali treatment of mitochondria or mitoplasts [Fujiki *et al.* (1982) as modified by van Loon and Young (1986)], for SDS–polyacrylamide gel electrophoresis on 13–14% polyacrylamide gels, and for fluorography (Gasser *et al.*, 1982), except that polyacrylamide gels containing *in vitro*-synthesized proteins were heated to 100°C for 5–10 min in 5% trichloroacetic acid, washed with water and neutralized with Tris-base before incubation with Na-salicylate. Autoradiography was done using pre-flashed films. Spectrophotometric quantitation of silver grains eluted from autoradiograms was done as described by Suissa (1983), except that the elution step was performed at 95°C for 10 min. The antibodies to cytochrome b_2 (Daum *et al.*, 1982a) and to citrate synthase (Riezman *et al.*, 1983) have been described.

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