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# Early Steps in Mitochondrial Protein Import: Receptor Functions Can Be Substituted by the Membrane Insertion Activity of Apocytochrome c

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## Summary

**The process of insertion of precursor proteins into mitochondrial membranes was investigated using a hybrid protein ($pSc_1$–c) that contains dual targeting information and, at the same time, membrane insertion activity. $pSc_1$–c is composed of the matrix-targeting domain of the cytochrome $c_1$ presequence joined to the amino terminus of apocytochrome c. It can be selectively imported along either a cytochrome $c_1$ route into the mitochondrial matrix or via the cytochrome c route into the intermembrane space. In contrast to cytochrome $c_1$, $pSc_1$–c does not require the receptor system/GIP for entry into the matrix. The apocytochrome c in the $pSc_1$–c fusion protein appears to exert its membrane insertion activity in such a manner that the matrix-targeting sequence gains direct access to the membrane potential–dependent step. These results attribute an essential function to the receptor system in facilitating the initial insertion of precursors into the mitochondrial membranes.**

## Introduction

Nuclear-encoded mitochondrial proteins are imported into mitochondria along complex pathways. These involve a series of steps that act in concert to efficiently and specifically direct proteins to their correct intracellular and intramitochondrial locations. Many of these steps are common for a large number of protein constituents (for reviews, see Nicholson and Neupert, 1988; Pfanner et al., 1988a; Hartl et al., 1989), which include: synthesis of precursor proteins with amino-terminal extension sequences (Hurt et al., 1984a, 1984b; Horwich et al., 1985; van Loon and Young, 1986; van Loon et al., 1986; Hartl et al., 1986, 1987); ATP-dependent maintenance of the precursor protein in a translocation-competent form (Pfanner et al., 1987; Chen and Douglas, 1987; Eilers and Schatz, 1986; Verner and Schatz, 1987); binding to specific receptor proteins on the mitochondrial surface (Zwizinski et al., 1983, 1984; Hennig et al., 1983; Riezman et al., 1983; Pfaller and Neupert, 1987); subsequent insertion of proteins into the outer mitochondrial membrane assisted by protein components (e.g., the putative general insertion protein, GIP) (Pfaller et al., 1988; Pfanner et al., 1988b); membrane potential–dependent insertion into or through the inner membrane via translocation contact sites (Schleyer et al., 1982; Pfanner and Neupert, 1985; Schleyer and Neupert, 1985; Schwaiger et al., 1987); removal of the amino-terminal presequence by the matrix processing peptidase enzyme (Böhni et al., 1983; Hawlitschek et al., 1988; Pollock et al., 1988); and intramitochondrial sorting and assembly into functional complexes assisted by hsp-60, a chaparonin-type protein complex (Cheng et al., 1989).

On the other hand, not all mitochondrial precursor proteins follow this elaborate pathway. It is interesting in this regard to compare the import of the two c-type cytochromes in mitochondria, namely cytochromes c and $c_1$, both of which are located on the outer surface of the inner membrane. The precursor of cytochrome $c_1$ ($pC_1$) contains a long amino-terminal prepiece that is processed in two steps upon import into mitochondria (Teintze et al., 1982; Gasser et al., 1982; Ohashi et al., 1982; Schleyer and Neupert, 1985; Sadler et al., 1984; Römisch et al., 1987). After initial binding to protease-sensitive surface receptors, $pC_1$ inserts into the outer membrane (Pfaller et al., 1988, and this work) and is then imported via contact sites (Schleyer and Neupert, 1985) into the mitochondrial matrix where it is processed to its intermediate form ($iC_1$). The second domain of the presequence (which bears similarity to bacterial leader sequences) directs the export of the protein across the inner membrane, a process termed "conservative sorting" (Hartl et al., 1986, 1987). The covalent attachment of heme to $iC_1$ occurs in a reaction catalyzed by cytochrome $c_1$ heme lyase (Nicholson et al., 1989) followed by the second proteolytic processing, producing the mature-sized cytochrome $c_1$ ($mC_1$) (Ohashi et al., 1982; Teintze et al., 1982; Nicholson et al., 1989).

The import pathway of cytochrome c is strikingly different from that of cytochrome $c_1$. Cytochrome c is synthesized as a precursor, known as apocytochrome c, which does not contain an amino-terminal prepiece (Zimmermann et al., 1979; Zitomer and Hall, 1976; Smith et al., 1979; Matsuura et al., 1981; Scarpulla et al., 1981; Stuart et al., 1987). No protease-sensitive components have been found on the surface of mitochondria to mediate apocytochrome c binding and import (Nicholson et al., 1988). Instead, apocytochrome c appears to insert partially into the mitochondrial outer membrane (Rietveld and de Kruijff, 1984; Dumont and Richards, 1984; Rietveld et al., 1985, 1986; Berkhout et al., 1987), where it is recognized and binds with high affinity to specific binding sites (Hennig and Neupert, 1981; Hennig et al., 1983; Nicholson et al., 1987) in a complex that includes cytochrome c heme lyase (CCHL) (Nicholson et al., 1988). Unlike all other mitochondrial precursors crossing the outer membrane studied to date, cytochrome c requires neither a membrane potential nor ATP for translocation (Zimmermann et al., 1981; Pfanner and Neupert, 1985; Nicholson and Neupert, unpublished data). Translocation into the intermembrane space is believed to be driven by the refolding of the polypeptide as a result of the covalent attachment of heme, a reaction catalyzed by the enzyme CCHL

(Hennig and Neupert, 1981; Nicholson et al., 1988; Dumont et al., 1988; Nicholson and Neupert, 1989).

The question thus arises as to how cytochrome c biogenesis has evolved to escape the "conservative sorting" pathway that cytochrome $c_1$ and other precursors must follow to gain access to the mitochondrial intermembrane space. We have examined the possibility that the unique property of apocytochrome c to spontaneously insert into membranes confers upon the precursor protein the ability to circumvent the early steps of mitochondrial import, namely, surface receptor binding and initial membrane insertion. We addressed this question by constructing a novel fusion protein composed of the matrix-targeting domain of the cytochrome $c_1$ presequence joined to the amino terminus of apocytochrome c. We have characterized the import pathways of this fusion protein, which contains dual targeting information for distinct import pathways. Furthermore, we have demonstrated that properties of the apocytochrome c molecule can substitute for a receptor/GIP system by directly mediating insertion of the precursor protein into the mitochondrial membrane system where the matrix-targeting domain can then respond to the membrane potential for complete translocation across the inner membrane into the matrix.

## Results

### The $pSc_1$–c Hybrid Protein Can Be Imported along Two Distinct Routes

Unlike cytochrome c import, transport of cytochrome $c_1$ into mitochondria is dependent on the presence of a membrane potential ($\Delta\Psi$) across the inner membrane. Formation of holocytochrome c from apocytochrome c, on the other hand, is dependent on NADH for the reduction of heme. We could therefore selectively activate either the cytochrome $c_1$ import pathway by the presence of $\Delta\Psi$, or the pathway for cytochrome c import by the presence of NADH.

In the presence of $\Delta\Psi$, the fusion protein, $pSc_1$–c, consisting of the matrix-targeting sequence of cytochrome $c_1$ and of apocytochrome c (Figure 1), was imported into mitochondria and was processed to its mature-sized form, $mSc_1$–c (Figure 2A, lane 2). When the chelators EDTA and o-phenanthroline were added to the import reaction, in order to inhibit the matrix-located processing peptidase, $Sc_1$–c could be accumulated in mitochondria as its precursor form $pSc_1$–c (Figure 2A, lane 1). Both of these species, namely $pSc_1$–c and $mSc_1$–c, accumulated in mitochondria in a $\Delta\Psi$-dependent fashion and were of the

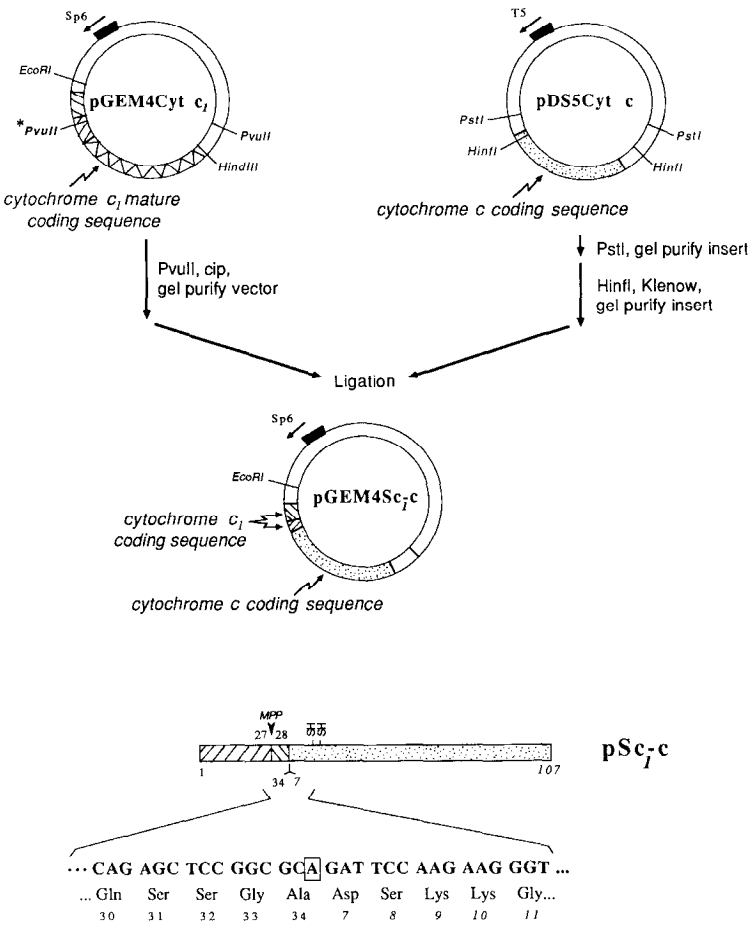


Figure 1. Construction of $pSc_1$–c, a Fusion Protein between Cytochrome $c_1$ and Cytochrome c

Details of the construction of the $pSc_1$–c fusion protein are described in Experimental Procedures. The unique PvuII site introduced into the cytochrome $c_1$ cDNA sequence by site-directed mutagenesis is indicated by an asterisk. $pSc_1$–c is a fusion protein between the first 34 amino acid residues of the cytochrome $c_1$ presequence and the complete sequence of cytochrome c, with the exception of the initial 6 amino acids of the amino terminus. This polypeptide is represented by a bar below. Hatched areas indicate sequences of cytochrome $c_1$ origin, while the dotted areas are of cytochrome c origin. Amino acids 1 through 27 represent the first portion of the cytochrome $c_1$ presequence that is removed by the matrix processing peptidase (MPP), which cleaves between amino acids 27 and 28. Amino acids 28 through 34 represent a small portion of the second part of the cytochrome $c_1$ presequence (7 amino acids). Amino acids 7 through 107 represent the cytochrome c part of the fusion polypeptide. Cysteine sulfhydryl residues to which the heme becomes attached are also indicated (-SH). The region around the fusion junction is enlarged, and details of the coding sequence, amino acid sequence, and amino acid numbering are indicated. The nucleotide introduced by mutagenesis is indicated by a box.

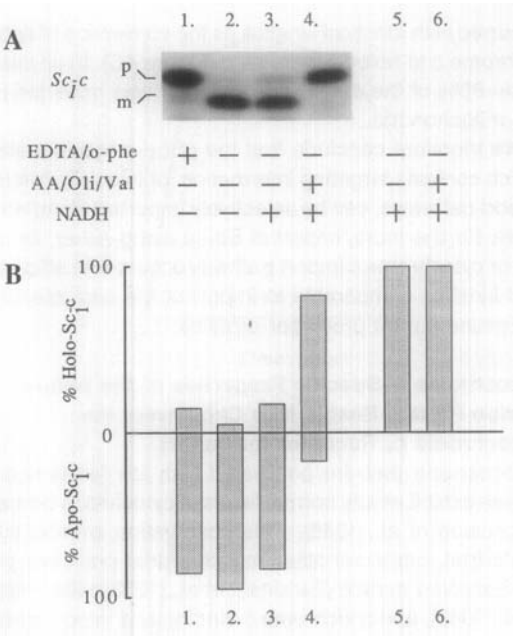


Figure 2. Import of $pSc_1$–c into Mitochondria

The precursor of $Sc_1$–c was synthesized in vitro in the presence of [$^{35}$S]cysteine and imported into mitochondria (100 μg of protein) at 25°C in the presence of 5 mM EDTA plus 0.2 mM o-phenanthroline (lane 1), 2.5 mM $MgCl_2$ (lanes 2, 3, and 4), 5 mM NADH (lanes 3 and 4), or antimycin A (AA), oligomycin (Oli), and valinomycin (Val) (lane 4) as described in Experimental Procedures. Following proteinase K treatment, samples were lysed in 1 ml of 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris–HCl (pH 7.2), containing 2 mM PMSF. Samples were divided in half and processed as follows. $Sc_1$–c was immunoprecipitated from both sets of samples; however, one set was prepared for SDS–polyacrylamide gels and resulting fluorographs are depicted in (A). The remaining set of immunoprecipitates were dissociated with urea, digested with trypsin, and subjected to reverse-phase HPLC. The amounts of apo- and holo-$Sc_1$–c were quantified by collecting the corresponding peptides and determining their radioactivity (B). [$^{35}$S]-cysteine-labeled apocytochrome c was incubated with mitochondria (50 μg) at 25°C in the presence of 5 mM NADH (lanes 5 and 6) or antimycin A, oligomycin, and valinomycin (lane 6). Following proteinase K treatment, levels of holocytochrome c formed were assayed (as described above for $Sc_1$–c) by immunoprecipitation and subsequent HPLC analysis.

heme-free apocytochrome c form (Figure 2B, lanes 1 and 2). In the absence of $\Delta\Psi$, import of $pSc_1$–c did not occur (data not shown) unless NADH was present. Under these conditions (i.e., $-\Delta\Psi$, +NADH), the cytochrome c pathway alone was active and a $pSc_1$–c species (Figure 2A, lane 4) to which heme was attached (Figure 2B, lane 4) accumulated in the mitochondria. When both the cytochrome $c_1$ (matrix import pathway) and the cytochrome c pathway were active (i.e., $+\Delta\Psi$, +NADH), the matrix route was dominant; larger amounts of protease-resistant $mSc_1$–c were accumulated than $pSc_1$–c (Figure 2A, lane 3). Reduction of import along the cytochrome c route under these conditions was also reflected by the low levels of holo-$pSc_1$–c that were synthesized (Figure 2B, lane 3). Thus, formation of holo-$pSc_1$–c was favored under conditions where $\Delta\Psi$ was absent (i.e., when the matrix import route was inactive) in contrast to holocytochrome c formation, which was entirely independent of $\Delta\Psi$ (Figure 2B, lanes 5 and 6).

It would appear from these results that the $pSc_1$–c fusion protein could be selectively imported along either the matrix or the cytochrome c import pathway. If indeed this was the case, we would expect $pSc_1$–c and $mSc_1$–c imported in a $\Delta\Psi$-dependent manner to be located in the mitochondrial matrix. Holo-$pSc_1$–c should, on the other hand, be located in the intermembrane space. To determine the submitochondrial localization of these various $Sc_1$–c species, import of [$^{35}$S]cysteine-labeled $pSc_1$–c into mitochondria was performed either in the presence of $\Delta\Psi$ and EDTA–o-phenanthroline (Figure 3A) or $\Delta\Psi$ alone (Figure 3B) or in the presence of NADH but the absence of $\Delta\Psi$ (Figure 3C). Mitochondria were reisolated and treated with increasing concentrations of digitonin to open sequentially the intermembrane space and matrix compartments, as judged by release of the respective marker enzymes, adenylate kinase and fumarase.

When the matrix import pathway alone was active (i.e., $+\Delta\Psi$), imported $pSc_1$–c (Figure 3A) and $mSc_1$–c (Figure 3B) were released by subsequent digitonin treatment in a manner very similar to fumarase. This indicates that imported $pSc_1$–c and $mSc_1$–c were soluble species and that they were located in the matrix of the mitochondrion. When the import of $pSc_1$–c was directed along the cytochrome c route (i.e., $-\Delta\Psi$, +NADH), the holo-$pSc_1$–c formed was resistant to added protease, which shows that it was translocated into the mitochondria however, it was found to be only partially soluble after digitonin treatment of the mitochondria. Apparently the presequence leads to an interaction of the uncleaved holo-form of the precursor with the mitochondrial membrane. Therefore, fractionation of the mitochondria with digitonin was combined with protease treatment. Holo-$pSc_1$–c was digested as soon as the intermembrane space was opened (Figure 3C). This demonstrates that the holo-$pSc_1$–c was located in the intermembrane space.

It has recently been described that nonspecific (i.e, nonmitochondrial) targeting signals appear to use a bypass pathway for import into mitochondria (Pfaller et al., 1988; Pfanner et al., 1988b). The kinetics and efficiencies of this bypass import are significantly reduced in comparison with those of legitimate mitochondrial precursor proteins. It was essential, therefore, to investigate the possibility that the import of $pSc_1$–c described above was a result of a low-efficiency nonspecific pathway(s). The kinetics of accumulation of $pSc_1$–c (Figure 4A) and $mSc_1$–c (Figure 4B) in the mitochondrial matrix were studied in parallel to those of the accumulation of the precursor form and the intermediate-sized form of cytochrome $c_1$ ($pC_1$ abd $iC_1$, respectively) for direct comparison. Likewise, the kinetics of holo-$pSc_1$–c formation were compared with the kinetics of holocytochrome c formation from apocytochrome c (Figure 4C). Accumulation of matrix-located $pSc_1$–c occurred with the same kinetics as $pC_1$ accumulation (Figure 4A). Similarly, import and processing to $mSc_1$–c by the matrix-located processing peptidase also displayed kinetics similar to $iC_1$ accumulation (Figure 4B). Formation of holo-$pSc_1$–c (i.e., along the cytochrome c import route)

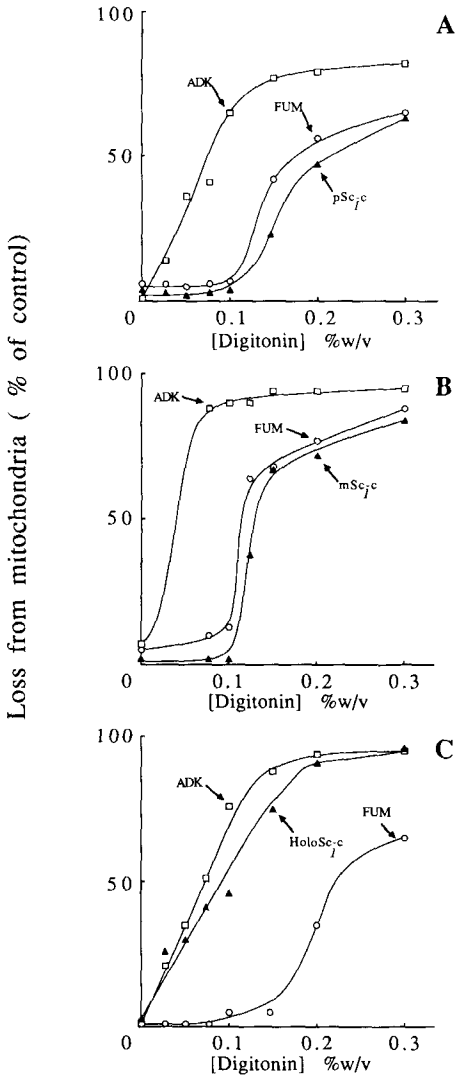


Figure 3. Submitochondrial Localization of Imported $Sc_1$–c Species by Digitonin Treatment of Mitochondria

The [$^{35}$S]cysteine-labeled $pSc_1$–c was imported into mitochondria (675 μg of protein, 2.25 ml final volume) at 25°C in the presence of 5 mM EDTA plus 0.2 mM o-phenanthroline (A), 2.5 mM $MgCl_2$ (B), or 5 mM NADH plus antimycin A, oligomycin, and valinomycin (C). Following proteinase K treatment, mitochondria were reisolated and resuspended in SEM buffer containing 100 mM KCl (SEMK) and divided into nine aliquots. Digitonin was added to concentrations of 0%–0.3% (w/v) and incubated for 2 min at 0°C in a final volume of 20 μl and at a protein concentration of 5 mg/ml. Afterwards, reactions were diluted 20-fold with SEMK and separated into pellet and supernatant fractions by centrifugation for 10 min at 27,000 × g (Beckman JA20 rotor). In the case of holo-$pSc_1$–c (C), prior to centrifugation, samples were treated with proteinase K (60 μg/ml, for 30 min at 0°C). PMSF (2 mM) was then added to inhibit further proteolytic activity. These samples were then treated identically to those in (A) and (B), which was as follows. Supernatants were adjusted to 1% Genapol (w/v) and the pellets were resuspended in SEMK plus 1% Genapol, to the same volume as the supernatants. Both supernatants and pellet fractions were divided in half. $Sc_1$–c was immunoprecipitated (see Experimental Procedures) from one set of samples and analyzed either by SDS–PAGE, where the resulting fluorographs were quantified by laser densitometry ([A] and [B], ▲) or the immunoprecipitates were digested with trypsin and subjected to reverse-phase HPLC. The holo-$pSc_1$–c peptides were collected and quantified by determining their radioactivity ([C], ▲). The remaining parts of the samples were used for determination of marker enzyme activities: adenylate kinase (□) and fumarase (○). For each digitonin concentration, the activities recovered from the pellet and supernatant fractions were set to 100%. The loss of enzyme activity or $Sc_1$–c protein from the mitochondria is plotted. ADK, adenylate kinase; FUM, fumarase.

occurred with identical kinetics as the conversion of apocytochrome c to holocytochrome c (Figure 4C). In all cases, 70%–80% of the employed precursor was imported into the mitochondria.

We therefore conclude that the $pSc_1$–c hybrid protein, which contains targeting information for two very different import pathways, can be selectively imported along either route. Furthermore, import of $Sc_1$–c along either the matrix or cytochrome c import pathway occurs with efficiency and kinetics comparable to import of the corresponding legitimate parent precursor proteins.

### Cytochrome c–Specific Properties of the $pSc_1$–c Fusion Protein Enable It to Circumvent the Cytochrome $c_1$ Receptor for Import

Mitochondria that are pretreated with low levels of proteases exhibit nearly normal levels of cytochrome c import (Nicholson et al., 1988). This observation strongly suggests that, unlike all other mitochondrial precursor proteins studied to date (Zwizinski et al., 1983, 1984; Pfaller et al., 1988), apocytochrome c binding and import are not mediated by protease-sensitive components on the surface of mitochondria (Nicholson et al., 1988). Thus, removal of the cytochrome $c_1$ receptor by mild proteinase K pretreatment of mitochondria enabled us to selectively inactivate the initial recognition step of the cytochrome $c_1$ import pathway, while at the same time leaving the entire cytochrome c pathway intact.

Mitochondria pretreated with proteinase K (50 μg/ml) were tested under various conditions for their ability, in comparison with untreated mitochondria, to import cytochrome $c_1$ and $pSc_1$–c (Figure 5). In the control mitochondria, the cytochrome $c_1$ precursor ($pC_1$) was imported into the mitochondrial matrix (Figure 5A, lane 1) where it underwent processing to intermediate-sized cytochrome $c_1$ ($iC_1$) (Figure 5A, lane 2) and was retranslocated back across the inner membrane (Hartl et al., 1987). In the presence of dithionite, conversion to holocytochrome $c_1$ took place followed by a second processing event (Nicholson et al., 1989), which resulted in the formation of mature-sized (holo) cytochrome $c_1$ ($mC_1$) (Figure 5A, lane 3). Import of cytochrome $c_1$, as previously reported, was clearly dependent on $\Delta\Psi$ (Figure 5A, lane 4). Removal of the cytochrome $c_1$ receptor by treatment of mitochondria with proteinase K abolished cytochrome $c_1$ import (Figure 5A, lanes 5–8). In the absence of the cytochrome $c_1$ receptor, import of $pSc_1$–c along the cytochrome c pathway occurred as expected (Figure 5B, lane 8 versus 4), but more interestingly, efficient import of $pSc_1$–c into the matrix, where it was processed to $mSc_1$–c, also occurred (Figure 5B, lane 5 versus 1 and lane 6 versus 2).

We conclude that properties of the hybrid protein (specifically the cytochrome c domain) or components of the

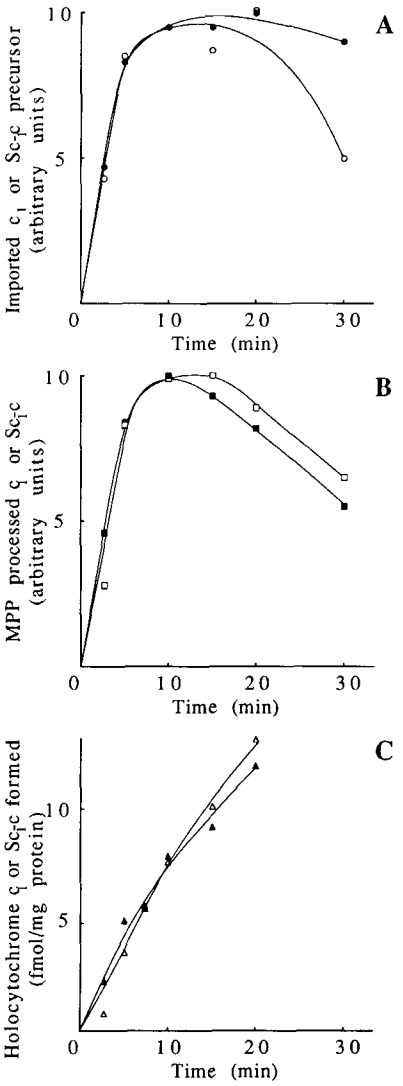


Figure 4. Kinetics of $pSc_1$–c Import into the Matrix and of Holo-$Sc_1$–c Formation

The cytochrome $c_1$ and $Sc_1$–c precursors were synthesized in vitro in the presence of [$^{35}$S]cysteine and then imported into mitochondria under conditions described for Figure 2, except that the incubation periods were varied.

(A) Accumulation of protease-resistant precursor-sized cytochrome $c_1$ and $Sc_1$–c ($pC_1$ and $pSc_1$–c): mitochondria (50 μg of protein) were incubated with [$^{35}$S]cysteine-labeled precytochrome $c_1$ (○) or $pSc_1$–c (●) at 25°C for the indicated time periods in the presence of EDTA plus o-phenanthroline. Protease-resistant $pC_1$ and $pSc_1$–c was then determined (see Experimental Procedures) following resolution by SDS–PAGE. Cytochrome $c_1$ and $Sc_1$–c bands from fluorographs of resulting gels were quantified by laser densitometry.

(B) Accumulation of protease-resistant processed cytochrome $c_1$ and $Sc_1$–c ($iC_1$ and $mSc_1$–c): import of precytochrome $c_1$ (□) and $pSc_1$–c (■) into mitochondria was carried out essentially as described in (A), except in the presence of 2.5 mM $MgCl_2$ instead of chelators. After incubation for the indicated time periods at 25°C, samples were processed exactly as in (A). MPP, matrix processing peptidase.

(C) Formation of holocytochrome c and holo-$pSc_1$–c in the presence of NADH: [$^{35}$S]cysteine-labeled apocytochrome c and apo-$pSc_1$–c were incubated (separately) with mitochondria (50 μg of protein) in the presence of 5 mM NADH at 25°C for the indicated time periods. Samples were cooled to 0°C and lysed with 1 ml of 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris/HCl (pH 7.2), and cytochrome c and $Sc_1$–c were immunoprecipitated (see Experimental Procedures). Following dissociation of the immune complexes with urea, peptides were generated by digestion with trypsin and resolved by reverse-phase HPLC. The amounts of holocytochrome c (△) and holo-$Sc_1$–c (▲) were quantified by collecting the corresponding peptides and determining their radioactivity.

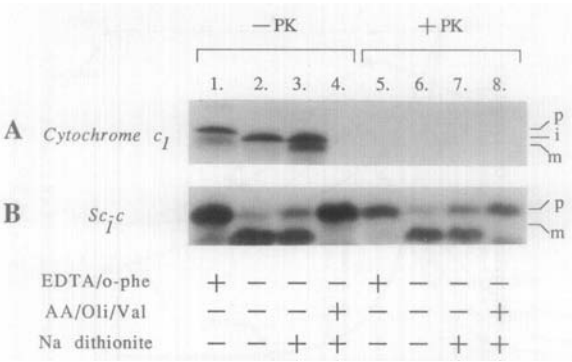


Figure 5. Removal of the Cytochrome $c_1$ Receptor by Protease Treatment of Mitochondria Does Not Affect Import of $pSc_1$–c into the Matrix

Freshly isolated mitochondria were treated with proteinase K at a concentration of 50 μg/ml as described in Experimental Procedures. Control mitochondria were processed in an identical manner except no protease was present. The resulting mitochondrial preparations were tested for their ability to import [$^{35}$S]methionine-labeled precytochrome $c_1$ (A) or $pSc_1$–c (B), essentially as described for Figure 2. Import into control (lanes 1–4) and protease-treated mitochondria (lanes 5–8) was performed in the presence of 5 mM EDTA plus 0.2 mM o-phenanthroline (lanes 1 and 5), 2.5 mM $MgCl_2$ (lanes 2–4 and 6–8), sodium dithionite (1 mg/ml) (lanes 3, 4, 7, and 8), or a mix of antimycin A (AA), oligomycin (Oli), and valinomycin (Val) (lanes 4 and 8). Protease-resistant species were analyzed by SDS–PAGE and fluorography. The bands corresponding to cytochrome $c_1$ or $Sc_1$–c in the resulting film are shown.

cytochrome c import pathway facilitate the cytochrome $c_1$ receptor–independent import of $Sc_1$–c into the mitochondrial matrix.

### $pSc_1$–c Does Not Require GIP for Import into the Mitochondrial Matrix

It has recently been reported that precursor proteins that are bound specifically to receptors on the mitochondrial surface are subsequently transferred to a putative component in the outer membrane called GIP, for general insertion protein (Pfaller et al., 1988). Transfer of precursors to this common site can be competed for by water-soluble porin (ws porin). Competition studies with ws porin can therefore be used for determining whether GIP is involved in the import of other precursor proteins. We thus addressed the question of whether receptor-independent import of $pSc_1$–c was in fact also GIP independent.

Water-soluble porin was prepared (Pfaller and Neupert, 1987) and tested for its ability to compete for the integration of [$^{35}$S]porin into the outer membrane, as a control, and for the import of cytochrome $c_1$ and $Sc_1$–c into the mitochondrial matrix (Figure 6). Water-soluble porin not only competed for the import of porin into the mitochondria but also for the import of cytochrome $c_1$ (as judged by $iC_1$ formation). Half-maximal inhibition of cytochrome $c_1$ import occurred at a concentration of ws porin of 90

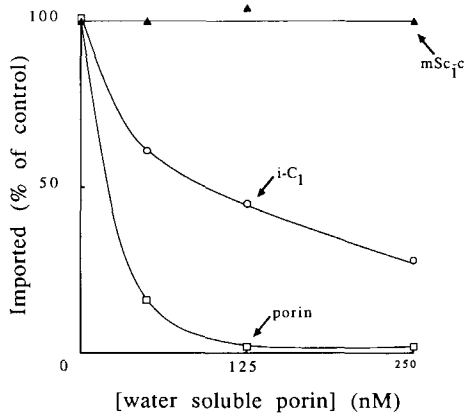


Figure 6. Import of $pSc_1$–c into the Mitochondrial Matrix Is Not Competed for by Water-Soluble Porin

The precursors of $Sc_1$–c, cytochrome $c_1$, and ws porin were synthesized in a reticulocyte lysate in the presence of [$^{35}$S]methionine. The various lysates (20 μl each) were incubated with isolated mitochondria (10 μg) and 50 μl of ws porin (dissolved in 0.1 M sodium phosphate [pH 6.8]; Pfaller and Neupert, 1987) at the concentrations indicated. The final volume was adjusted to 200 μl with BSA-containing buffer. Samples were incubated for 40 min at 0°C, after which antimycin A, oligomycin, and valinomycin were added to final concentrations of 8 μM, 20 μM, and 0.5 μM, respectively. Mitochondria were recovered by centrifugation and resuspended in 100 μl of BSA-containing buffer and incubated for a further 15 min at 25°C. Samples were then cooled to 0°C, and in the case of ws porin were treated with 200 μg of proteinase K per ml, whereas those for $Sc_1$–c and cytochrome $c_1$ were treated with 20 μg of proteinase K per ml. All samples were incubated for 30 min at 0°C. Protease activity was halted by the addition of 1 ml of SEM buffer containing 2 mM PMSF. Mitochondria were reisolated by centrifugation and lysed in SDS-containing buffer, and all samples were resolved on SDS–polyacrylamide gels. Imported porin (□), $iC_1$ (○), and $mSc_1$–c (▲) were quantified by subsequent laser densitometry of fluorographs of the resulting gels.

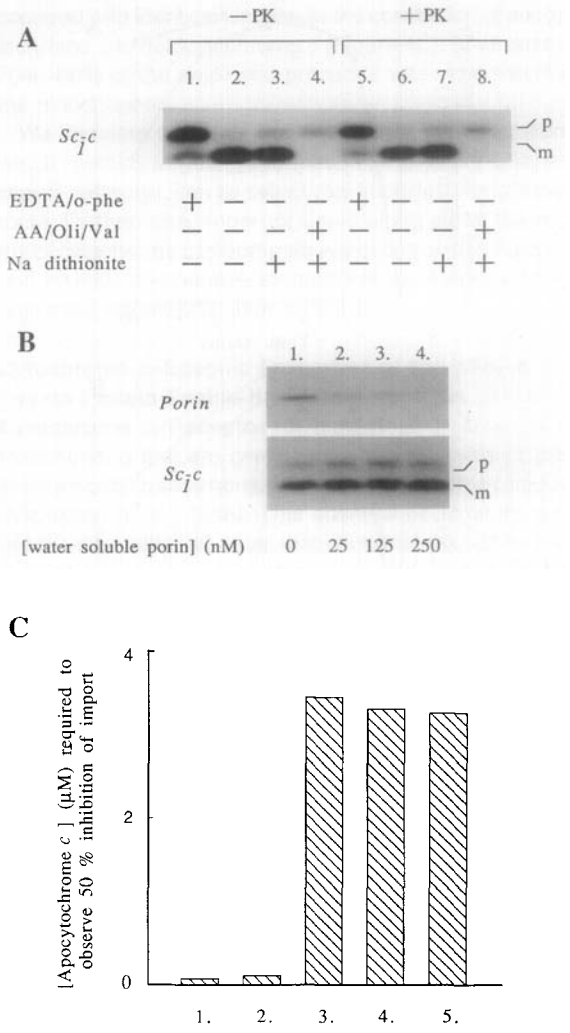


Figure 7. Import of $pSc_1$–c into *cyt2-1* Mitochondria and Competition for Import into Wild-Type Mitochondria

(A) Isolated *cyt2-1* mitochondria were either untreated (lanes 1–4) or treated with proteinase K (lanes 5–8) at a concentration of 50 μg/ml as outlined in Experimental Procedures. Import of [$^{35}$S]$pSc_1$–c was performed exactly as described in the legend to Figure 5, except that mutant *cyt2-1* mitochondria were used instead of wild-type mitochondria. The import of cytochrome $c_1$ into *cyt2-1* mitochondria was identical to its import into wild-type mitochondria, as shown in Figure 5.

(B) [$^{35}$S]$pSc_1$–c and porin were imported into *cyt2-1* mitochondria in the presence of increasing concentrations of ws porin, essentially as described in the legend to Figure 6. Following the chase reaction for 15 min at 25°C, import of porin was assessed by protease resistance following treatment with 200 μg of proteinase K per ml. Import of $pSc_1$–c into the matrix was monitored by $mSc_1$–c formation. Mitochondria were lysed with SDS-containing buffer, and all samples were resolved on SDS–PAGE. The bands corresponding to protease-resistant porin and to $Sc_1$–c in the resulting films are shown.

(C) $^{35}$S-labeled apocytochrome c, cytochrome $c_1$, and $pSc_1$–c were imported into wild-type mitochondria under various conditions in the presence of increasing concentrations of unlabeled apocytochrome c (at the concentrations indicated) as described in Experimental Procedures. Formation of holocytochrome c (lane 1) and holo-$pSc_1$–c (lane 2) and accumulation of $iC_1$ (lane 3) and $mSc_1$–c (lane 4) and $mSc_1$–c in protease-pretreated mitochondria (lane 5) were quantified. The concentrations of apocytochrome c required to observe 50% inhibition of import of the precursor proteins are presented.

pmol/ml, thus indicating that cytochrome $c_1$ import into the matrix was dependent on GIP. Import of $pSc_1$–c into the matrix (as measured by $mSc_1$–c accumulation), however, was clearly not competed for by ws porin.

Taken together, these data indicate that $pSc_1$–c can be imported into the mitochondrial matrix independent of both the cytochrome $c_1$ receptor and the common insertion site (GIP), both of which are essential for cytochrome $c_1$ import.

### Receptor/GIP-Independent Import of $pSc_1$–c into the Matrix Is Not Assisted by Components of the Cytochrome c Import Machinery

Is the observed receptor/GIP-independent import of $pSc_1$–c into the matrix somehow assisted by components of the cytochrome c import pathway, for example CCHL? It has previously been proposed that CCHL alone may account for both major stages of cytochrome c import, namely, specific binding of apocytochrome c and its conversion to holocytochrome c (Nicholson et al., 1988). Therefore, to examine the possible role of CCHL in the import of $pSc_1$–c into the matrix, two approaches were taken.

In a first approach, we employed the *cyt2-1* mutant of Neurospora crassa, the mitochondria of which are unable

to bind apocytochrome c with high affinity and are deficient in CCHL activity (Nargang et al., 1988). A productive cytochrome c import pathway therefore does not exist in the *cyt2-1* mitochondria. The import of cytochrome $c_1$ into *cyt2-1* mitochondria, like the import of all other precursor proteins tested, was completely unaffected (Nargang et al., 1988). Import of $pSc_1$–c into the matrix of *cyt2-1* mitochondria occurred with a similar efficiency as its import into wild-type mitochondria (data not shown). Similarly, $pSc_1$–c import into the matrix of protease-treated *cyt2-1* mitochondria (Figure 7A, lanes 5–7) was comparable to its import into untreated mitochondria (Figure 7A, lanes 1–3). Import of $pSc_1$–c into *cyt2-1* mitochondria along the cytochrome c pathway was, however, severely blocked (Figure 7A, lanes 4 and 8). Therefore, in the absence of both cytochrome c receptor and a functional cytochrome c import apparatus, $pSc_1$–c was still able to enter the mitochondrial matrix with high efficiency.

As in wild-type mitochondria, ws porin successfully blocked import at the level of GIP in isolated *cyt2-1* mitochondria, as judged by the inhibition of the import of labeled porin (Figure 7B, upper panel). The *cyt2-1* mitochondria could still import $pSc_1$–c into the matrix, where it was processed to $mSc_1$–c, even when GIP was occupied by ws porin (Figure 7B, lower panel).

In a second approach, competition for cytochrome c import components was tested using unlabeled apocytochrome c to see if the receptor-independent import of $pSc_1$–c into the matrix was impeded (Figure 7C). As a control, $^{35}$S-labeled apocytochrome c and precytochrome $c_1$ were incubated with isolated mitochondria (separately) in the presence of varying concentrations of unlabeled apocytochrome c. Holocytochrome c formation (from [$^{35}$S]-apocytochrome c) was competed by the added apocytochrome c; half-maximal competition was observed at an apocytochrome c concentration of about 100 nM (see also Hennig et al., 1983). Import of cytochrome $c_1$ and processing to $iC_1$, however, were not competed by similar concentrations of apocytochrome c (Figure 7C). Unlabeled apocytochrome c could not specifically compete for the $\Delta\Psi$-dependent import of $pSc_1$–c and processing to $mSc_1$–c in either untreated or proteinase K–treated mitochondria (Figure 7C). This observation could be accounted for if the $Sc_1$–c precursor has a higher affinity for the cytochrome c import pathway than apocytochrome c. Import of $pSc_1$–c along the cytochrome c route (as measured by holo-$pSc_1$–c formation) is, however, specifically competed by apocytochrome c, indicating that $pSc_1$–c has about the same affinity as the legitimate apocytochrome c molecule (Figure 7C). Apparently, the import of $pSc_1$–c into the mitochondrial matrix was not assisted by the proteinaceous components of the cytochrome c import pathway.

We then asked whether features of the apocytochrome c molecule itself might account for the unusual ability of $pSc_1$–c to circumvent the receptor/GIP system. Most notable among these features is the ability of apocytochrome c to spontaneously insert into membranes. Subtle changes in apocytochrome c conformation (i.e., following freeze/thaw cycles) affect both its membrane insertion activity (Rietveld et al., 1985) and its subsequent binding and import competence (Hennig and Neupert, 1983). Similarly, modulation of apocytochrome c conformation by nucleoside triphosphate (NTP)-dependent processes also affects binding and import; the more folded (membrane active) form of apocytochrome c, generated in vitro by NTP depletion, is also more import competent than its unfolded (membrane inactive) counterpart (Nicholson and Neupert, unpublished data). $pSc_1$–c import into the mitochondrial matrix responded to NTP-modulated conformational changes in a manner identical to holocytochrome c formation from apocytochrome c (Figure 8). Depletion of NTPs by apyrase pretreatment resulted in a more folded $pSc_1$–c (as judged by resistance to proteinase K digestion) (Figure 8, insert) that in turn was more efficiently imported into the mitochondrial matrix. In contrast, ATP added to import mixtures inhibited $pSc_1$–c import (whereas nonhydrolyzable analogs did not) concomitant with NTP-dependent unfolding of the precursor molecule (data not shown). For all other proteins studied thus far, NTP-dependent unfolding is requisite for entry into the matrix. $pSc_1$–c, on the other hand, contravenes this normal behavior and a partially folded conformation supports import into the matrix. We conclude that the membrane insertion properties of the apocytochrome c part of the $pSc_1$–c, which are highly dependent on the apocytochrome c conformational state

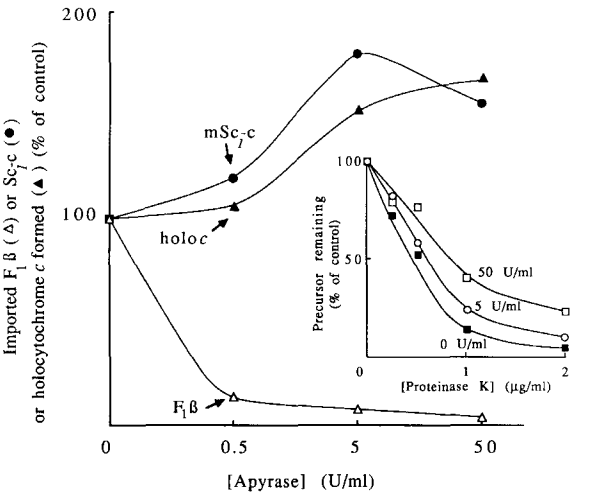


Figure 8. Depletion of ATP Levels Stimulates Import of $pSc_1$–c into the Matrix

ATP was depleted from reticulocyte lysates containing radiolabeled precursors ($pSc_1$–c, apocytochrome c, and the precursor to $F_1$-ATPase subunit β) as described in Experimental Procedures, and import into isolated mitochondria was analyzed. The amounts of holocytochrome c and of protease-resistant $mF_1\beta$ and $mSc_1$–c are expressed as a percentage of the control to which no apyrase was added. Insert: Depletion of ATP from reticulocyte lysate containing in vitro synthesized $pSc_1$–c, decreases $pSc_1$–c sensitivity to proteinase K. Portions of the apyrase-treated $pSc_1$–c reticulocyte lysate described above were incubated with proteinase K at the indicated concentrations for 30 min at 0°C. The amount of undigested precursor protein remaining (expressed as a percentage of the control to which no proteinase K was added) was determined following SDS–PAGE, fluorography, and laser densitometry, as described in Experimental Procedures. The concentrations of apyrase used for the pretreatment of the reticulocyte lysate are indicated.

and can be modulated by NTP addition or withdrawal, must be maintained for the early stages by which $pSc_1$–c circumvents both receptor/GIP and the cytochrome c import machinery.

In support of this, fusion proteins consisting of a matrix targeting sequence equivalent to that of cytochrome $c_1$ (from ATPase subunit 9 or cytochrome $b_2$) and of a passenger protein without membrane insertion properties (such as mouse dihydrofolate reductase [DHFR] or $F_1$-ATPase subunit β) were found to use the receptor/GIP pathway (Pfaller et al., 1988, and unpublished data).

### When Bound to Mitochondria, $pSc_1$–c Can Migrate between the Import Machineries for the Separate Routes

We next asked if there was any overlap between the two pathways by initiating $pSc_1$–c import on the cytochrome c pathway and then attempting to divert it onto a route leading to the matrix. Mitochondria were treated with proteinase K to remove the cytochrome $c_1$ receptor and with antimycin A plus oligomycin to inhibit the formation of a membrane potential (Figure 9). Under these conditions, $pSc_1$–c was bound to mitochondria without any further import along either the cytochrome c pathway (because of the absence of NADH or dithionite) or the matrix pathway (because of the absence of $\Delta\Psi$). The mitochondria containing bound $pSc_1$–c were washed and reisolated. Then $pSc_1$–c was chased along either the cytochrome c pathway by adding sodium dithionite (Figure 9A) or the matrix pathway by reestablishing a membrane potential (Figure 9B). Upon the addition of sodium dithionite, bound $pSc_1$–c was converted to holo-$pSc_1$–c with high efficiency (Figure 9A, lane 1 versus lane 2). If KCl (0.3 M) was added following the binding incubation but prior to the chase, subsequent formation of holo-$pSc_1$–c during the chase was unaffected (Figure 9A, lane 3). This indicates that about 70% of the bound $pSc_1$–c was initially present in a salt-resistant complex with CCHL and had entered a cytochrome c pathway (such a complex with CCHL has been described in detail for apocytochrome c by Nicholson et al. [1988]). On the other hand, over 50% of the bound $pSc_1$–c could also be chased into the mitochondrial matrix, where it was processed to $mSc_1$–c (Figure 9B, lane 4 versus lane 5). This route was severely impeded by the presence of salt (Figure 9B, lane 6). In the initial binding incubation (–receptor, –NADH, $-\Delta\Psi$) approximately 70% of $pSc_1$–c had entered the cytochrome c import pathway, and yet over 50% of mitochondria-associated $pSc_1$–c could be chased to $mSc_1$–c if the $\Delta\Psi$ was reestablished. Therefore, at least some of the $Sc_1$–c that was imported into the matrix was derived from that which was initially in the salt resistant complex with cytochrome c heme lyase.

In summary, these findings indicate that $pSc_1$–c bound to mitochondria existed in an equilibrium from which it could be chased along either one of two distinct import pathways. The majority of the bound $pSc_1$–c had entered the cytochrome c import pathway, but a large proportion of it could be rerouted to the matrix upon reestablishing a membrane potential. We conclude, therefore, that initiation of a precursor along one mitochondrial import pathway (i.e., the cytochrome c route) does not necessarily commit it to that pathway. As this indeed is the case, it implies that a functional intersection or exchange between cytochrome c and matrix-import pathways can exist.

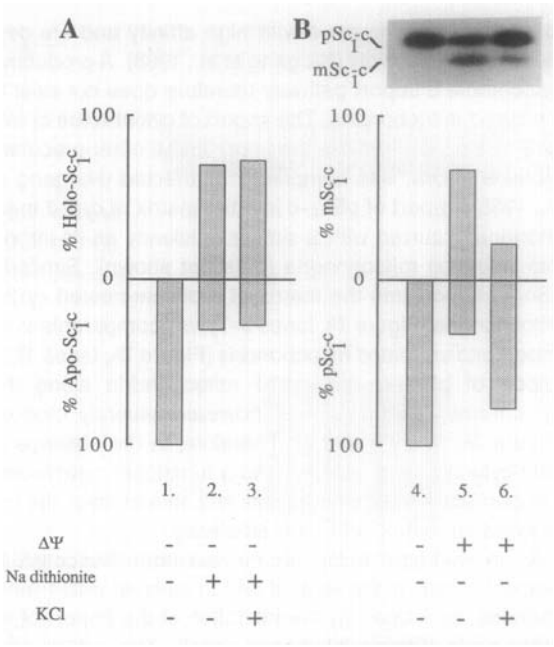


Figure 9. $pSc_1$–c Bound to Protease-Treated Mitochondria Can Be Imported by Either Matrix or Cytochrome c Pathways

[$^{35}$S]cysteine-labeled $pSc_1$–c (75 μl of reticulocyte lysate) was bound to protease-pretreated mitochondria (300 μg of protein, in a final volume of 600 μl in SME buffer) in the presence of holocytochrome c (from horse heart), antimycin A, and oligomycin for 10 min at 0°C. The mitochondria were reisolated by centrifugation and resuspended in fresh SEM buffer. The bound $pSc_1$–c was chased along either the cytochrome c or matrix pathway in mixtures equivalent to 50 μg of mitochondrial protein (containing bound $pSc_1$–c) in a final volume of 100 μl of BSA buffer.

(A) Chase along the cytochrome c pathway. Samples were incubated for 20 min at 25°C in the presence of either antimycin A, oligomycin, and valinomycin (lanes 1, 2, and 3), sodium dithionite (1 mg/ml) (lanes 2 and 3), or KCl (0.3 M final concentration). Total cytochrome c was immunoprecipitated from the samples, and the amount of apo- and holo-$Sc_1$–c was determined by HPLC analysis of tryptic peptides as described in Experimental Procedures. Results are given as the percentage of the total mitochondrial-associated cytochrome c present as either apo-$Sc_1$–c or holo-$Sc_1$–c.

(B) Chase along the matrix-import pathway. $pSc_1$–c was chased to mature-sized $Sc_1$–c ( $mSc_1$–c) by incubation for 20 min at 25°C either in the presence of antimycin A, oligomycin, and valinomycin (lane 4) or in the presence of 0.2 mM N,N,N′,N′-tetramethylphenylenediamine plus 8 mM potassium ascorbate (from a stock adjusted to pH 7.2 with KOH) to reestablish a membrane potential (lanes 5 and 6) or in the presence of 0.3 M KCl (lane 6). All samples contained 2.5 mM $MgCl_2$ to activate the matrix processing peptidase. Following incubation, mitochondria were reisolated, lysed in SDS-containing buffer, and resolved by SDS–PAGE. the $Sc_1$–c bands from a fluorograph of the resulting gel are shown. The bands were quantified by laser densitometry, and the percentage of precursor (% $pSc_1$–c) and processed mature-sized (% proteins $mSc_1$–c) are given.

## Discussion

In an attempt to understand the process of translocation

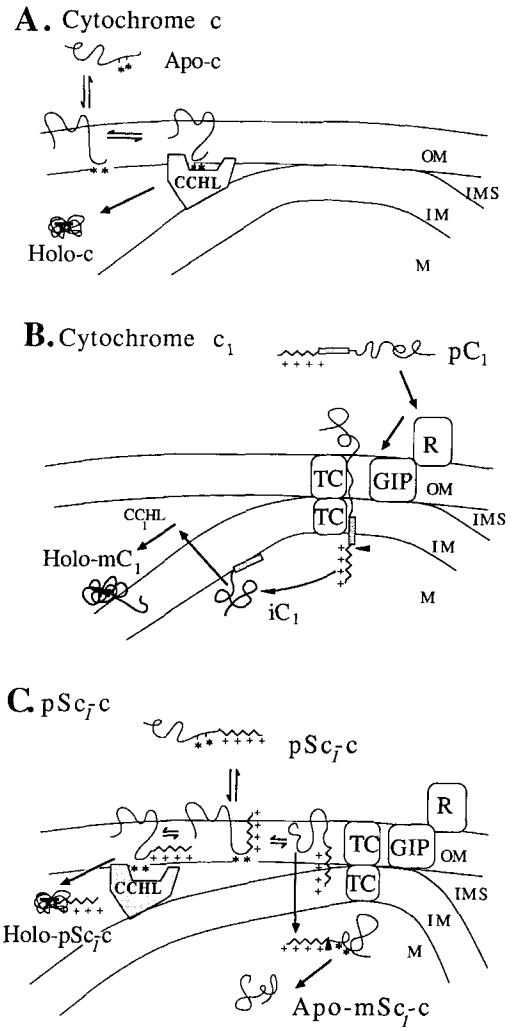


Figure 10. Hypothetical Import and Assembly Pathways of Cytochrome c, Cytochrome $c_1$, and p$Sc_1$–c

(A) Apocytochrome c (Apo-c), the precursor of holocytochrome c (holo-c), partially inserts into the mitochondrial outer membrane (OM) where it binds to cytochrome c heme lyase (CCHL), which acts as a specific binding site in lieu of a surface receptor. Translocation across the outer membrane directly into the intermembrane space (IMS) is believed to be driven by the refolding of the cytochrome c polypeptide as a result of covalent heme addition. Cysteine sulfhydryl residues to which the heme becomes attached are indicated by asterisks.

(B) After synthesis in the cytosol, the precursor of cytochrome $c_1$ ($pC_1$) binds specifically to distinct receptor sites (R) on the mitochondrial surface. Following insertion into the outer membrane (OM), mediated by the general insertion protein (GIP), $pC_1$ is translocated through contact sites (translocation contact sites, TC) into the matrix (M), in a membrane-dependent manner. Processing to an intermediate-sized cytochrome $c_1$ ($iC_1$) occurs followed by retranslocation across the inner membrane (IM). Conversion of cytochrome $c_1$, thus exposed to the intermembrane space (IMS) to its holo counterpart, is catalyzed by cytochrome $c_1$ heme lyase ($CC_1HL$). The second processing event, resulting in the formation of holo mature-sized cytochrome $c_1$ (Holo-m$C_1$), then ensues.

(C) p$Sc_1$–c initially inserts into the mitochondrial outer membrane (OM) owing to the membrane-active nature of its cytochrome c domain. Bound p$Sc_1$–c exists in an equilibrium from which it can be chased along either a cytochrome c- or matrix-import pathway. Upon the addition of a reductant, bound p$Sc_1$–c could be imported along the cytochrome c pathway, where it would accumulate in the intermembrane space (IMS) as a heme-containing species, holo-p$Sc_1$–c. On the other hand, bound p$Sc_1$–c could be chased into the mitochondrial matrix (M) in a membrane potential–dependent manner, where it would be processed to its mature-sized form (m$Sc_1$–c) and would accumulate as a soluble species in the matrix (Apo-m$Sc_1$–c).

of precursor proteins across mitochondrial membranes, we have employed a hybrid protein that contains membrane insertion activity. The hybrid protein, p$Sc_1$–c, composed of the matrix-targeting domain of the cytochrome $c_1$ presequence joined to the amino terminus of apocytochrome c, contains dual targeting information for distinct mitochondrial import pathways and can be selectively imported along either a cytochrome $c_1$ route into the mitochondrial matrix or via the cytochrome c pathway into the intermembrane space. The proposed import pathways of p$Sc_1$–c are depicted in the model in Figure 10C, where the import pathways of cytochrome c (Figure 10A) and of cytochrome $c_1$ (Figure 10B) are also outlined for comparison.

We have shown here that the membrane insertion activity of apocytochrome c, which is active in p$Sc_1$–c, can aid the insertion of a mitochondrial targeting sequence attached to its amino terminus in such a manner that the targeting sequence responds to $\Delta\Psi$ and directs the apocytochrome c–containing fusion protein into the matrix. It is likely, therefore, that the apocytochrome c part of the p$Sc_1$–c constitutes the structural element responsible for the initial recognition and interaction of p$Sc_1$–c with outer mitochondrial membranes. In this way the p$Sc_1$–c hybrid protein is able to circumvent the receptor/GIP-mediated steps of import into the matrix. How then does p$Sc_1$–c gain access to the $\Delta\Psi$-requiring steps leading to translocation into the matrix? In all likelihood, the early events of p$Sc_1$–c membrane insertion involve only an interaction between the precursor protein and the lipids of the outer membrane, as described for apocytochrome c. The first step in this mechanism is an electrostatic interaction between positively charged amino acids within apocytochrome c and anionic phospholipids on the outer surface of the outer membrane. These bound anionic phospholipids then appear to be sequestered into a microenvironment surrounding the apocytochrome c molecule. During or following this interaction, α-helical structure is expressed within the apocytochrome c polypeptide and its amino terminus is then inserted approximately halfway through the lipid bilayer (Rietveld and de Kruijff, 1984; Rietveld et al., 1985, 1986; Jordi et al., 1989). At this point during the import of cytochrome c, apocytochrome c gains access to specific proteinaceous components of the cytochrome c import machinery (i.e., CCHL) (Nicholson et al., 1988). By a similar initial interaction with anionic membrane phospholipids, the apocytochrome c component of p$Sc_1$–c may mediate penetration of the hybrid far enough through the mitochondrial membrane system to become responsive to $\Delta\Psi$ and thereby continue translocation along this route into the matrix. Taken together, these findings give insight into several important features of import of proteins into mitochondria.

First, our results allow us to differentiate two major processes in the translocation of proteins across the mito-

chondrial membranes. In the first process, proteins become inserted into the outer mitochondrial membrane in such a fashion that they acquire competence for transport across the inner membrane. In a second process, the precursors are translocated across the inner membrane, a reaction that requires the membrane potential, $\Delta\Psi$, for initiation. This raises the interesting question of whether the first process, insertion of the presequence into the outer membrane, is a reaction that is dispensable when precursors get direct access to the inner membrane, i.e., in an artifactual system comprised of isolated inner membrane vesicles. Most interestingly, $pSc_1$–c does not seem to use translocation contact sites for transfer across the inner membrane. A fusion protein consisting of the first 167 amino acids of cytochrome $b_2$ and the complete DHFR protein was found to block contact sites in the presence of methotrexate, which leads to tight folding of the DHFR domain, which in turn causes a blockage at these import sites. With large amounts of the purified fusion protein expressed in E. coli, import into the matrix of all precursor proteins tested could be inhibited, indicating that the authentic precursors all use the same translocation contact sites. The import of the fusion protein $pSc_1$–c into the mitochondrial matrix, however, was not blocked (unpublished data). This suggests that $pSc_1$–c, once inserted into the outer membrane, does not necessarily enter translocation contact sites but finds its way across the inner membrane in a different and unknown way.

Second, our findings explain why all mitochondrial precursor proteins (except cytochrome c) examined thus far require a receptor/GIP system for import. Thus, a caveat arises for fusion proteins or modified (i.e., truncated) proteins that may artifactually possess membrane insertion activity and thereby bypass receptors (Pfanner et al., 1988b).

Third, the pathway for import into mitochondria that is dependent on apocytochrome c insertion properties is not separate or exclusive of the $\Delta\Psi$-dependent pathway. Fusion proteins containing signals for both can move between these pathways and are directed one way or the other in a "tug-of-war" type of reaction. In the case of $pSc_1$–c, the $\Delta\Psi$-dependent pathway to the matrix is dominant over the cytochrome c pathway into the intermembrane space when both routes are fully active during ongoing import. The ability of $pSc_1$–c to initiate import along the cytochrome c pathway but then be redirected to the mitochondrial matrix along the $\Delta\Psi$-dependent pathway demonstrates that some functional intersection of these pathways exist that bridges the exchange of $Sc_1$–c from one pathway to the other under appropriate conditions.

Fourth, the membrane insertion properties of apocytochrome c may explain in part how the cytochrome c import pathway has evolved in such a way as to have escaped conservative sorting and receptor/GIP-mediated events. Spontaneous insertion into the mitochondrial membrane may be the first step in cytochrome c translocation. There may be no specificity of this step for mitochondrial outer membranes, or there may be limited specificity (e.g., exerted by the particular lipid composition). Only in the case of the mitochondria, however, is there a specific recognition and tight binding involving CCHL. This then appears to be followed by covalent heme attachment, and then folding-dependent translocation across the outer membrane accounts for the absence of a requirement for a surface receptor and GIP during import. In contrast, because the mature part of cytochrome $c_1$ does not have membrane insertion activity, both a receptor and GIP are required for specific recognition and membrane insertion. Once initiated along this route, cytochrome $c_1$ then apparently requires transit along a conservative sorting pathway (i.e., via the matrix) in order to become properly assembled in the intermembrane space.

The striking difference in the mechanisms by which cytochromes c and $c_1$ are imported into the intermembrane space seems to reflect the divergence of their assembly pathways during the evolution of mitochondria. It is possible that the major factor contributing to this divergence is the difference in their respective membrane insertion properties.

## Experimental Procedures

### DNA Manipulations and Construction of the $pSc_1$–c Fusion Protein

Isolation of DNA fragments and oligonucleotides, plasmid preparations, ligation, phosphorylation, and dephosphorylation of DNA, transformation, and DNA sequencing were performed as previously described (Maniatis et al., 1982; Müller and Zimmermann, 1988; Stuart et al., 1987). Restriction enzymes, calf intestinal phosphatase, T4 DNA ligase, Klenow fragment of E. coli DNA polymerase, and SP6 RNA polymerase were from Boehringer Mannheim. T4 polynucleotide kinase was from New England Biolabs.

For the construction of the plasmids encoding the fusion protein $Sc_1$–c (Figure 1), the EcoRI–HindIII fragment of N. crassa cytochrome $c_1$ DNA containing the 5′ noncoding region and the amino-terminal half of the cytochrome $c_1$ protein (Römisch et al., 1987) was cloned into the plasmid pMa5–8 (Kramer et al., 1984). Oligonucleotide site-directed mutagenesis (essentially as described by Müller and Zimmermann, 1988) was performed to introduce a unique PvuII site in the cytochrome $c_1$ cDNA sequence by changing a C residue for an A residue at a position corresponding to codon 34. DNA restriction analysis and sequencing were performed to verify correct mutagenesis. The mutagenized EcoRI–HindIII fragment was cloned into the polylinker of the plasmid pGEM4. The recombinant plasmid was then subsequently cleaved with PvuII and treated with calf intestinal phosphatase and purified by gel electroelution.

A PstI cDNA insert containing the complete coding region of cytochrome c was isolated following cleavage of the corresponding pDS5 plasmid (Stuart et al., 1987). The purified insert was further cleaved with HinfI (which cuts the insert at a position corresponding to codon 7 and again in the 3′ noncoding region) and was treated with the Klenow fragment of E. coli DNA polymerase I. The resulting insert was purified and ligated into the pGEM4 vector described above to produce an in-frame fusion between the cytochrome $c_1$ and cytochrome c fragments. DNA sequencing was carried out to verify the postulated nucleotide sequence at the join region. The corresponding amino acid sequence, which contains no introduced residues, is depicted in Figure 1. The fusion protein was synthesized in a reticulocyte lysate following transcription of the plasmids with SP6 RNA polymerase, as previously described (Nicholson et al., 1988).

The matrix processing peptidase cleavage site of the cytochrome $c_1$ presequence was determined as follows. The $pSc_1$–c fusion protein was synthesized in reticulocyte lysate in the presence of [$^{35}$S]cysteine and was imported into isolated mitochondria (as described below) in the presence of $MgCl_2$. Protease-resistant mature-sized fusion protein ($mSc_1$–c) was purified by immunoprecipitation and subjected to radiosequence analysis (Wachter et al., 1973; Hartl et al., 1986) (data not

shown). The proposed cleavage site determined by this method occurs between amino acids 27 and 28 of the cytochrome $c_1$ presequence and is indicated by an arrowhead in Figure 1.

### Cell Growth and Subcellular Fractionation

N. crassa (wild type 74A) was grown at 25°C for 14–16 hr with vigorous aeration and bright illumination as previously described (Hennig and Neupert, 1983). The cyt2-1 mutant (Nargang et al., 1988) was grown under identical conditions except that cultures were incubated for 40–48 hr. Mitochondria were isolated by differential centrifugation essentially as described before (Pfanner and Neupert, 1985), except in a buffer containing 250 mM sucrose, 2 mM EDTA, 10 mM MOPS–KOH (pH 7.2) (SEM buffer) plus 2 mM PMSF added from a freshly prepared stock solution in ethanol. PMSF was omitted from SEM buffer in experiments where protease treatment was involved.

### Import of Cytochrome c and Measurement of Holocytochrome c Formation

Transcription of apocytochrome c cDNA cloned into pGEM3 was carried out as previously described (Nicholson et al., 1988). Apocytochrome c was then synthesized in nuclease-treated rabbit reticulocyte lysate (Pelham and Jackson, 1976) in the presence of L-[$^{35}$S]cysteine (1100–1400 Ci/mmol, Amersham) as previously described (Nicholson et al., 1988). Aliquots of postribosomal supernatants were stored at −80°C under nitrogen gas.

Unless otherwise indicated, binding and import of apocytochrome c were performed as follows: mitochondria (50 μg) were incubated in a mixture (final volume of 100 μl) containing BSA buffer (3% [w/v] bovine serum albumin, 70 mM KCl, 220 mM sucrose, 10 mM MOPS–KOH [pH 7.2]), 3 μM hemin, and 15 μl of [$^{35}$S]cysteine-labeled reticulocyte lysate. The reaction was started by adding NADH (5 mM final concentration) or sodium dithionite (freshly prepared to a final concentration 1 mg/ml) (Nicholson and Neupert, 1989) and incubating at 25°C for 15 min. Reducing agents were omitted in cases where apocytochrome c binding, but not holocytochrome c formation, was required. The reaction mixture was then lysed by adding 1 ml of buffer containing 1% (w/v) Triton X-100, 0.3 M NaCl, and 10 mM MOPS–KOH (pH 7.2). Samples were immunoprecipitated with antisera directed against both apo- and holocytochrome c. The amounts of apocytochrome c and synthesized holocytochrome c were determined by HPLC analysis of [$^{35}$S] cysteine-containing tryptic peptides essentially as described by Nicholson et al. (1988).

### Import of Cytochrome $c_1$ and $pSc_1$–c into Mitochondria

The precursors of cytochrome $c_1$ and $pSc_1$–c were synthesized in rabbit reticulocyte lysate in the presence of L-[$^{35}$S]methionine or [$^{35}$S]cysteine (Amersham), as indicated. Synthesis was directed by transcripts from pGEM plasmids as previously described (Hartl et al., 1987). Postribosomal supernatants were prepared and supplemented according to Zimmermann and Neupert (1980). Aliquots of the lysates were stored at −80°C.

Unless otherwise indicated, mitochondria (50 μg) were incubated for 20 min at 25°C in a mixture (total volume of 100 μl) containing 3% (w/v) bovine serum albumin, 70 mM KCl, 220 mM sucrose, 2.5 mM $MgCl_2$, 10 mM MOPS–KOH (pH 7.2), and 15 μl of $^{35}$S-labeled precursor reticulocyte lysate. Where indicated, the matrix-localized processing peptidase was inhibited by the addition of 5 mM EDTA and 0.2 mM o-phenanthroline (Schmidt et al., 1984). Dissipation of the mitochondrial membrane potential ($\Delta\Psi$) was achieved by the addition of a mixture of antimycin A, oligomycin, and valinomycin (added from a 100-fold concentrated stock solution in ethanol), to final concentrations of 8 μM, 20 μM, and 0.5 μM, respectively. Following incubation, the import mixtures were cooled to 0°C and treated with 20 μg of proteinase K per ml (from Tritrachium album; Boehringer) for 30 min at 0°C. The cytochrome $c_1$ and $pSc_1$–c precursors in reticulocyte lysate and the mitochondrial accumulated species in the presence of 1% (w/v) Triton X-100 were completely protease sensitive to the concentrations of proteinase K used (data not shown). For analysis of cytochrome $c_1$ import, samples were diluted with SEM buffer containing 2 mM PMSF; mitochondria were reisolated by centrifugation at 17,400 × g for 12 min (Beckman JA-20 rotor), lysed with SDS-containing sample buffer, and resolved by SDS–PAGE. For analysis of $Sc_1$–c import, proteinase K activity was stopped by the addition of 1 ml of 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM MOPS–KOH (pH 7.2) containing 2 mM PMSF. Samples were immunoprecipitated using an antiserum directed against cytochrome c (i.e., apo- plus holocytochrome c). Immunoprecipitates were analyzed either by HPLC to quantify apo- and holopeptides or by SDS–PAGE. In all cases, radioactive bands in the dried acrylamide gels were visualized by fluorography and quantified by laser densitometry of the resulting film. The amounts of protease-resistant cytochrome $c_1$ or $Sc_1$–c are expressed in arbitrary units.

### Pretreatment of Mitochondria with Protease

Freshly isolated mitochondria were suspended in 1.0 ml of SEM buffer at a protein concentration of 1 mg/ml and cooled to 0°C. Proteinase K was added (from a 1 mg/ml stock solution) to a concentration of 50 μg/ml and incubated at 0°C for 30 min. Protease activity was then inhibited by the addition of 1.0 ml of SEM buffer containing 1 mM PMSF. After a further 5 min incubation at 0°C, the mitochondria were reisolated by centrifugation at 17,400 × g for 12 min. The mitochondrial pellet was then resuspended in fresh SEM buffer at a concentration of 2 mg/ml. No residual protease activity was detected following this treatment (data not shown).

### Miscellaneous Methods

Published methods were used for the following: preparation of holocytochrome c and apocytochrome c, and production of specific antibodies in rabbits (Hennig and Neupert, 1983); immunoprecipitation and preparation of samples for SDS–PAGE (Nicholson et al., 1987); SDS–PAGE (Laemmli, 1970); protein determination (Bradford, 1978); radioactivity determination, fluorography (Nicholson et al., 1987), and treatment of mitochondria with digitonin (Hartl et al., 1987); treatment of reticulocyte lysates with apyrase and determination of proteolytic sensitivity of precursor proteins (Pfanner et al., 1987; Nicholson and Neupert, unpublished data); measurement of adenylate kinase activity (Schmidt et al., 1984) and measurement of fumarase activity (Kanarek and Hill, 1964); radiosequencing analysis by automated solid-phase Edman degradation (Wachter et al., 1973); and competition studies with ws porin (Pfaller and Neupert, 1987; Pfaller et al., 1988) or with apocytochrome c (Hennig et al., 1983).

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### References

Berkhout, T. A., Rietveld, A., and de Kruijff, B. (1987). Preferential lipid association and mode of penetration of apocytochrome *c* in mixed model membranes as monitored by tryptophanyl fluorescence quenching using brominated phospholipids. Biochem. Biophys. Acta *897*, 1–4.

Böhni, P. C., Daum, G., Leaver, C., and Schatz, G. (1983). Import of proteins into mitochondria. Partial purification of a matrix-located protease involved in the cleavage of mitochondrial precursor peptides. J. Biol. Chem. *258*, 4937–4943.

Bradford, M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.

Chen, W.-J., and Douglas, M. G. (1987). Phosphodiester bond cleavage outside mitochondria is required for the completion of protein import into the mitochondrial matrix. Cell *49*, 651–658.

Cheng, M. Y., Hartl, F.-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L., and Horwich, A. (1989). Mitochondrial heat shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. Nature *337*, 620–625.

Dumont, M. E., and Richards, F. M. (1984). Insertion of apocytochrome *c* into lipid vesicles. J. Biol. Chem. *248*, 4147–4156.

Dumont, M. E., Ernst, J. F., and Sherman, F. (1988). Coupling of heme attachment to import of cytochrome *c* into yeast mitochondria. J. Biol. Chem. *263*, 15928–15937.

Eilers, M., and Schatz, G. (1986). Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. Nature *322*, 228–232.

Gasser, S. M., Ohashi, A., Daum, G., Böhni, P. C., Gibson, J., Reid, G. A., Yonetani, T., and Schatz, G. (1982). Import of proteins into mitochondria: energy-dependent uptake of precursors by isolated mitochondria. Proc. Natl. Acad. Sci. USA *79*, 267–271.

Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H., and Neupert, W. (1986). Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of the ubiquinol–cytochrome c reductase. Cell *47*, 939–951.

Hartl, F.-U., Ostermann, J., Guiard, B., and Neupert, W. (1987). Successive translocation into and out of the mitochondrial matrix: targeting of proteins to the intermembrane space by a bipartite signal peptide. Cell *51*, 1027–1037.

Hartl, F.-U., Pfanner, N., Nicholson, D. W., and Neupert, W. (1989). Mitochondrial protein import. Biochim. Biophys. Acta *998*, 1–45.

Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U., and Neupert, W. (1988). Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein. Cell *53*, 795–806.

Hennig, B., and Neupert, W. (1981). Assembly of cytochrome *c*. Apocytochrome *c* is bound to specific sites on mitochondria before its conversion to holocytochrome *c*. Eur. J. Biochem. *121*, 302–312.

Hennig, B., and Neupert, W. (1983). Biogenesis of cytochrome *c* in *Neurospora crassa*. Meth. Enzymol. *97*, 261–274.

Hennig, B., Köhler, H., and Neupert, W. (1983). Receptor sites involved in the posttranslational transport of apocytochrome *c* into mitochondria. Proc. Natl. Acad. Sci. USA *80*, 4963–4967.

Horwich, A. L., Kalousek, F., Mellman, I., and Rosenberg, L. E. (1985). A leader peptide is sufficient to direct mitochondrial import of a chimeric protein. EMBO J. *4*, 1129–1135.

Hurt, E. C., Pesold-Hurt, B., and Schatz, G. (1984a). The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihydrofolate reductase into the mitochondrial matrix. EMBO J. *3*, 3149–3156.

Hurt, E. C., Pesold-Hurt, B., and Schatz, G. (1984b). The cleavage prepiece of an imported mitochondrial protein is sufficient to direct cytosolic dihydrofolate reductase into yeast mitochondrial matrix. FEBS Lett. *178*, 306–310.

Jordi, W., Li-Xin, Z., Pilon, M., Demel, R. A., and de Kruijff, B. (1989). The importance of an amino terminus of the mitochondrial precursor protein apocytochrome *c* for translocation across model membranes. J. Biol. Chem. *264*, 2292–2301.

Kanarek, L., and Hill, R. L. (1964). The preparation and characterisation of fumarase from swine heart muscle. J. Biol. Chem. *239*, 4202–4206.

Kramer, W., Drutsa, D., Jansen, H.-W., Kramer, B., Pflugfelder, M., and Fritz, H. J. (1984). The gapped duplex DNA approach to oligonucleotide-directed mutation construction. Nucl. Acids Res. *12*, 9441–9456.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Matsuura, S., Arpin, M., Hannum, C., Margoliash, E., Sabatini, D. D., and Morimoto, T. (1981). *In vitro* synthesis and posttranslational uptake of cytochrome *c* into isolated mitochondria: role of a specific addressing signal in the apocytochrome. Proc. Natl. Acad. Sci. USA *78*, 4368–4372.

Müller, G., and Zimmermann, R. (1988). Import of prepromelittin into the endoplasmic reticulum: energy requirements for membrane insertion. EMBO J. *7*, 639–648.

Nargang, F. E., Drygas, M. E., Kwong, P. L., Nicholson, D. W., and Neupert, W. (1988). A mutant of *Neurospora crassa* deficient in cytochrome *c* heme lyase activity cannot import cytochrome *c* into mitochondria. J. Biol. Chem. *263*, 9388–9394.

Nicholson, D. W., and Neupert, W. (1988). Synthesis and assembly of mitochondrial proteins. In Protein Transfer and Organelle Biogenesis, R. C. Das and P. W. Robbins, eds. (New York: Academic Press), pp. 677–746.

Nicholson, D. W., and Neupert, W. (1989). Import of cytochrome *c* into mitochondria requires reduced heme. Proc. Natl. Acad. Sci. USA *84*, 4340–4344.

Nicholson, D. W., Köhler, H., and Neupert, W. (1987). Import of cytochrome *c* into mitochondria: cytochrome *c* heme lyase. Eur. J. Biochem. *164*, 147–157.

Nicholson, D. W., Hergersberg, C., and Neupert, W. (1988). Role of cytochrome *c* heme lyase in the import of cytochrome *c* into mitochondria. J. Biol. Chem. *263*, 19034–19042.

Nicholson, D. W., Stuart, R. A., and Neupert, W. (1989). Biogenesis of cytochrome $c_1$: role of cytochrome $c_1$ heme lyase and of the two proteolytic processing steps during import into mitochondria. J. Biol. Chem. *264*, 10156–10168.

Ohashi, A., Gibson, J., Gregor, I., and Schatz, G. (1982). Import of proteins into mitochondria: the precursor of cytochrome $c_1$ is processed in two steps, one of them heme dependent. J. Biol. Chem. *257*, 13042–13047.

Pelham, H. R. B., and Jackson, R. J. (1976). An efficient mRNA dependent translational system from reticulocyte lysates. Eur. J. Biochem. *67*, 247–256.

Pfaller, R., and Neupert, W. (1987). High-affinity binding sites involved in the import of porin into mitochondria. EMBO J. *6*, 2635–2642.

Pfaller, R., Steger, H., Rassow, J., Pfanner, N., and Neupert, W. (1988). Import pathways of precursor proteins into mitochondria: multiple receptor sites are followed by a common membrane insertion site. J. Cell Biol. *107*, 2483–2490.

Pfanner, N., and Neupert, W. (1985). Transport of proteins into mitochondria: a potassium diffusion potential is able to drive the import of ADP/ATP carrier. EMBO J. *4*, 2819–2825.

Pfanner, N., Tropschug, M., and Neupert, W. (1987). Mitochondrial protein import: nucleoside triphosphates are involved in conferring import-competence to precursors. Cell *49*, 815–823.

Pfanner, N., Hartl, F.-U., and Neupert, W. (1988a). Import of proteins into mitochondria: a multi-step process. Eur. J. Biochem. *175*, 205–212.

Pfanner, N., Pfaller, R., and Neupert, W. (1988b). How finicky is mitochondrial protein import? Trends Biochem. Sci. *13*, 165–167.

Pollock, R. A., Hartl, F.-U., Cheng, M. Y., Ostermann, J., Horwich, A., and Neupert, W. (1988). The processing peptidase of yeast mitochondria: the two co-operating components MPP and PEP are structurally related. EMBO J. *7*, 3493–3500.

Rietveld, A., and de Kruijff, B. (1984). Is the mitochondrial precursor protein apocytochrome *c* able to pass a lipid bilayer? J. Biol. Chem. *259*, 6704–6707.

Rietveld, A., Ponjee, G. A. E., Schiffers, P., Jordi, W., van de Coolwijk, P. J. F. M., Demel, R. A., Marsh, D., and de Kruijff, B. (1985). Investigations on the insertion of the mitochondrial precursor protein apocytochrome *c* into model membranes. Biochim. Biophys. Acta *818*, 398–409.

Rietveld, A., Jordi, W., and de Kruijff, B. (1986). Studies on the lipid dependency and mechanisms of the translocation of the mitochondrial precursor protein apocytochrome *c* across model membranes. J. Biol. Chem. *261*, 3846–3856.

Riezman, H., Hay, R., Witte, C., Nelson, N., and Schatz, G. (1983). Yeast mitochondrial outer membrane specifically binds cytoplasmi-

cally synthesized precursors of mitochondrial proteins. EMBO J. *2*, 1113–1118.

Römisch, J., Tropschug, M., Sebald, W., and Weiss, H. (1987). The primary structure of cytochrome $c_1$ from *Neurospora crassa*. Eur. J. Biochem. *164*, 111–115.

Sadler, I., Suda, K., Schatz, G., Kaudewitz, F., and Haid, A. (1984). Sequencing of the nuclear gene for the yeast cytochrome $c_1$ precursor reveals an unusually complex amino-terminal presequence. EMBO J. *3*, 2137–2143.

Scarpulla, R. C., Agne, K. M., and Wu, R. (1981). Isolation and structure of a rat cytochrome *c* gene. J. Biol. Chem. *256*, 6480–6486.

Schleyer, M., and Neupert, W. (1985). Transport of proteins into mitochondria: translocation intermediaters spanning contact sites between outer and inner membrane. Cell *43*, 339–350.

Schleyer, M., Schmidt, B., and Neupert, W. (1982). Requirement of a membrane potential for the posttranslational transfer of proteins into mitochondria. Eur. J. Biochem. *125*, 109–116.

Schmidt, B., Wachter, E., Sebald, W., and Neupert, W. (1984). Processing peptidase of *Neurospora crassa*. Two step cleavage of imported ATPase subunit 9. Eur. J. Biochem. *144*, 581–588.

Schwaiger, M., Herzog, V., and Neupert, W. (1987). Characterization of translocation contact sites involved in the import of mitochondrial proteins. J. Cell Biol. *105*, 235–246.

Smith, M., Leung, D. W., Gillam, S., Astell, C. R., Montgomery, D. L., and Hall, B. D. (1979). Sequence of the gene for iso-1-cytochrome *c* in *Saccharomyces cerevisiae*. Cell *16*, 753–761.

Stuart, R. A., Neupert, W., and Tropschug, M. (1987). Deficiency in mRNA splicing in a cytochrome *c* mutant of *Neurospora crassa*: importance of the carboxy-terminus for the import of apocytochrome *c* into mitochondria. EMBO J. *6*, 2131–2137.

Teintze, M., Slaughter, M., Weiss, H., and Neupert, W. (1982). Biogenesis of the mitochondrial ubiquinol:cytochrome *c* reductase (cytochrome $bc_1$ complex). J. Biol. Chem. *257*, 10364–10371.

van Loon, A. P. G. M., and Young, E. T. (1986). Intracellular sorting of alcohol dehydrogenase isozymes in yeast: a cytosolic location reflects absence of an amino-terminal targeting sequence for the mitochondrion. EMBO J. *5*, 161–165.

van Loon, A. P. G. M., Brandli, A. W., and Schatz, G. (1986). The presequences of two imported mitochondrial proteins contain information for intracellular and intramitochondrial sorting. Cell *44*, 801–812.

Verner, K., and Schatz, G. (1987). Import of an incompletely folded precursor protein into isolated mitochondria requires an inner energized membrane, but no ATP. EMBO J. *6*, 2449–2456.

Wachter, E., Machleidt, W., Hofner, H., and Otto, J. (1973). Aminopropyl glass and its phenylene diisothiocyanate derivatives, a new support in solid-phase Edman degradation of peptides and proteins. FEBS Lett. *35*, 97–102.

Zimmermann, R., and Neupert, W. (1980). Transport of proteins into mitochondria. Posttranslational transfer of ADP/ATP carrier into mitochondria. Eur. J. Biochem. *109*, 217–229.

Zimmermann, R., Paluch, U., and Neupert, W. (1979). Cell-free synthesis of cytochrome *c*. FEBS Lett. *108*, 141–146.

Zimmermann, R., Hennig, B., and Neupert, W. (1981). Different transport pathways of individual precursor proteins into mitochondria. Eur. J. Biochem. *116*, 455–460.

Zitomer, R. S., and Hall, B. D. (1976). Yeast cytochrome *c* messenger RNA. J. Biol. Chem. *251*, 6320–6326.

Zwizinski, C., Schleyer, M., and Neupert, W. (1983). Transfer of proteins into mitochondria: precursor to the ADP/ATP carrier binds to receptor sites on isolated mitochondria. J. Biol. Chem. *258*, 4071–4074.

Zwizinski, C., Schleyer, M., and Neupert, W. (1984). Proteinaceous receptors for the import of mitochondrial precursor proteins. J. Biol. Chem. *259*, 7850–7856.