Import of Apocytochrome c into the Mitochondrial Intermembrane Space along a Cytochrome c\textsubscript{1} Sorting Pathway*

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Mitochondria contain two distinct c-type cytochromes, namely, cytochrome c and cytochrome c\textsubscript{1}. Apart from the similarity in their functions as electron carriers, cytochrome c and cytochrome c\textsubscript{1} share many features in common. Both of these proteins are encoded for in the nucleus, are synthesized in the cytosol, and are imported into mitochondria in a post-translational manner. Both proteins have their functional location at the outer surface of the inner membrane, and both contain protoheme IX as their prosthetic group. Despite these similarities, however, the import pathways of both of these cytochromes are drastically different. Cytochrome c\textsubscript{1}, in contrast to cytochrome c, uses a much more elaborate and complex pathway to gain access to the mitochondrial intermembrane space.

Cytochrome \textit{c} \textsubscript{1} is initially synthesized as a precursor protein (precytochrome c\textsubscript{1} or pCr) which contains a long amino-terminal bipartite presequence (Ohashi et al., 1982; Teintze et al., 1982; Sadler et al., 1984; Römisch et al., 1987; Hartl et al., 1987). Precytochrome c\textsubscript{1} binds specifically to receptor proteins on the outer surface of the mitochondrial outer membrane (Zwizinski et al., 1984; Schleyer and Neupert, 1985). Recently, in Neurospora crassa, this receptor protein has been identified as a protein of 19 kDa (MOM19), and it has been demonstrated to act as a receptor protein for a large subset of mitochondrial precursor proteins (Söllner et al., 1989). The precursor protein then becomes inserted into the mitochondrial outer membrane. This process is mediated by a proteinaceous component in the outer membrane which is used by all precursor proteins tested (except by cytochrome c) and is known as the general insertion protein (GIP)\textsuperscript{1} (Pfanner et al., 1988; Pfanner et al., 1990). The acquisition or maintenance of such a translocation-competent conformation requires the presence of nucleoside triphosphates (NTPs) (Pfanner and Neupert, 1986; Pfanner et al., 1987a). Subsequent transfer of precytochrome c\textsubscript{1} from GIP into the inner membrane occurs at regions in which the outer and inner membranes are in close contact (translocation contact sites), and this step requires an electrical membrane potential (\Delta \Psi) across the inner membrane (Schleyer and Neupert, 1985). Precytochrome c\textsubscript{1}, contrary to the "stop transfer" model, is initially transported into the mitochondrial matrix where it is processed to its intermediate size form (iCr\textsubscript{1}) by the matrix processing peptidase, whose activity is enhanced by another protein, the processing enhancing protein (van Loon et al., 1986; Hartl et al., 1987; Hauflitschek et al., 1988). The remaining carboxyl region of the presequence of cytochrome c\textsubscript{1}, is thought to direct the "export" of the protein across the inner membrane and thereby becomes exposed to the intermembrane space (Hartl et al., 1987). The heme group then becomes covalently attached to the iCr\textsubscript{1}, a process that is catalyzed by cytochrome c\textsubscript{1} heme lyase and which requires the presence of NADH and flavin nucleotides. Heme addition is a prerequisite for the second proteolytic processing event of cytochrome c\textsubscript{1}, which results in the production of mature size holocytochrome c\textsubscript{1} (Teintze et al., 1982; Nicholson et al., 1989). The complex assembly pathway used by cytochrome c\textsubscript{1} may be justified on the basis of the "conservative sorting principle" (Hartl et al., 1986, 1987; Hartl and Neupert, 1990). It is generally accepted that mitochondria evolved from prokaryotic ancestors that became introduced into a host cell following an endosymbiote event. During the evolution of mitochondria, prokaryotic principles of membrane assembly and protein sorting have, however, been conserved. The import pathways of many mitochondrial intermembrane space proteins have evolved so as to make use of the evolutionary remnants of preexisting "ancestral assembly pathways" of the bacterial endosymbiont. For example, cytochrome c\textsubscript{1} is initially imported into the mitochondrial matrix (a compartment comparable to the bacterial cytoplasm), and from there it is exported across the inner membrane along its ancestral pathway into the intermembrane space (a compartment comparable to the bacterial mitochondrial intermembrane space).

\textsuperscript{1}The abbreviations used are: GIP, general insertion protein; PMSF, phenylmethylsulfonyl fluoride; Mops, 4-morpholinepropanesulfonic acid; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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periplasm); this process is termed conservative sorting (Hartl and Neupert, 1980).

The assembly pathway of cytochrome c, in contrast, is relatively simple. The precursor protein, apocytochrome c, does not contain a cleavable presequence; consequently, the mitochondrial targeting information must reside in the mature sequence of the protein (Stewart et al., 1971; Zimmermann et al., 1981; Stuart et al., 1987). Specific binding of the precursor to mitochondria is not mediated by a protease-sensitive receptor on the external surface of the mitochondrial outer membrane (Nicholson et al., 1988). In contrast to all other mitochondrial precursor proteins, however, apocytochrome c is very membrane active and can insert spontaneously into the mitochondrial outer membrane (Rietveld et al., 1983, 1985a, 1985b, 1986; Berkhour et al., 1987; Jordi et al., 1989). The specificity of this process for lipid bilayers is low; however, only in the case of mitochondrial membranes can apocytochrome c bind with high affinity, by forming a complex with cytochrome c heme lyase (Nicholson et al., 1988). Cytochrome c heme lyase is the enzyme that is responsible for catalyzing the covalent heme attachment to apocytochrome c, thus resulting in holocytocrome c (Nargang et al., 1988; Nicholson et al., 1988; Dumont et al., 1987; Drygas et al., 1989). It would appear that cytochrome c heme lyase has a dual function in the import pathway of apocytochrome c, namely, as an initial high affinity binding site of the incoming precursor protein as well as the enzyme catalyzing its conversion to holocytocrome c (Nargang et al., 1988; Stuart and Neupert, 1990). Although cytochrome c heme lyase is distinct from cytochrome c1 heme lyase, it also displays a dependence on NADH and flavin nucleotides (Nicholson and Neupert, 1989). In the absence of reductants, heme addition does not take place; instead, apocytochrome c remains in a salt-resistant complex with cytochrome c heme lyase, however, still accessible to externally added proteases (Nicholson et al., 1988). Therefore, in contrast to the situation for apocytochrome c1, translocation of apocytochrome c across the membrane resulting in exposure to the intermembrane space does not occur prior to heme addition. Translocation of cytochrome c across the outer membrane does not require external energy sources such as a membrane potential or ATP2 (Zimmermann et al., 1981). Instead, it is postulated that the energy derived from folding of the polypeptide around its newly acquired heme group is the driving force for traversing the membrane (Nicholson et al., 1988; Nicholson and Neupert, 1980). Cytochrome c1, on the other hand, is sorted in a non-conservative manner, thus reflecting a divergence of the assembly pathways of the c-type cytochromes during evolution of mitochondria (Stuart et al., 1990; Stuart and Neupert, 1990).

We decided to address the question of whether cytochrome c could be functionally sorted along a conservative sorting pathway. In order to do this, a fusion protein pLC-c was constructed which contained the entire bipartite presequence of cytochrome c1 joined to the amino terminus of apocytochrome c. This hybrid protein thereby contained a 3-fold targeting information, namely, the cytochrome c domain for targeting across the outer membrane into the intermembrane space, a matrix targeting domain for import into the matrix, and finally the postulated reexport domain to direct export from the matrix across the inner membrane. We demonstrate here that the three targeting informations are indeed active and pLC-c could be imported selectively along a cytochrome c route into the intermembrane space or along a cytochrome c1 pathway, also into the intermembrane space, via a conservative sorting mechanism through the matrix. When delivered to the intermembrane space in a conservative sorting manner, however, holo formation of LC-c could not occur. We demonstrate that despite the apparent similarity of structure and functional location of the heme lyases and structural similarity of the heme binding regions in their respective apoproteins, cytochrome c heme lyase and cytochrome c1 heme lyase have nonoverlapping substrate specificities.

**MATERIALS AND METHODS**

**DNA Manipulations and Construction of the pLC-c Fusion Protein**

Preparation of plasmids, isolation of DNA inserts, DNA ligation, phosphorylation and dephosphorylation, transformation, and DNA sequencing were performed essentially as described before (Maniatis et al., 1982; Stuart et al., 1987, 1990). All restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase, calf intestinal phosphatase, and SP6 RNA polymerase were obtained from Boehringer Mannheim.

The fusion protein pLC-c is comprised of the complete presequence of cytochrome c1 joined to the amino terminus of apocytochrome c (see Fig. 1). The construction of the plasmid encoding pLC-c was performed in a manner identical to that described previously for pLSc-c (Stuart et al., 1990) with the exception that the unique PciI site introduced by site-directed mutagenesis in the cytochrome c1 cDNA sequence was achieved by changing a cysteine residue for an adenine at a position corresponding to codon 73. pLC-c is a fusion protein consisting of the entire presequence of cytochrome c1 plus 3 amino acids of the mature sequence fused to the complete sequence of apocytochrome c, with the exception of the initial 6 amino acids of the amino terminus. The fusion protein was synthesized and translated in E. coli lysate following transcription of the resulting recombinant pGEM4 plasmids with SP6 RNA polymerase, as described previously (Nicholson et al., 1988; Stuart et al., 1990).

**Cell Growth and Subcellular Fractionation**

*Neurospora crassa* (wild-type 74A) was grown for 15 h at 25°C with vigorous aeration and bright illumination as described previously (Hennig and Neupert, 1980). The *N. crassa* cyto2-1 mutant (Nargang et al., 1988) was grown under identical conditions with the exception that the cultures were incubated for 40–48 h. The hyphas were harvested by filtration, and mitochondria were isolated by differential centrifugation, essentially as described before (Pfanner and Neupert, 1983) in S buffer (250 mM sucrose, 2 mM EDTA, 10 mM Mops/KOH, pH 7.3) plus 2 mM PMSF. PMSF was omitted from the S buffer in experiments in which protease pretreatment of the mitochondria was involved.

**Import of Precursor Proteins into Mitochondria**

**Synthesis of Precursor Proteins**—Full-length cDNA coding for apocytochrome c, cytochrome c1, the Fe-S protein from the b/cl subcomplex, P-ATPase subunit β (Fβ), porin, pSc-c, and pLC-c cloned into pGEM plasmids were transcribed with SP6 RNA polymerase as described previously (Stuart et al., 1987, 1990; Hartl et al., 1987; Pfaller et al., 1988; Kleeve et al., 1987). The capped transcripts were then used to direct protein synthesis. The precursor proteins were synthesized in nuclease-treated rabbit reticulocyte lysates (Pelham and Jackson, 1986) in the presence of either L-[35S]cysteine or L-[35S]methionine (Amersham Corp.) as indicated, as described previously (Nicholson et al., 1987). The transcribed supernatants were prepared and supplemented according to Zimmermann and Neupert (1980), and aliquots of the lysates were stored at −80°C under nitrogen gas.

**Import of Apocytochrome c and Measurement of Holocytochrome c Formation**—Unless otherwise indicated, import of apocytochrome c was performed as follows. Mitochondria (75 µg of protein) were incubated in a mixture containing 200 µl of buffer containing 3 mM HEPES-NaOH buffer, 3 µM hemin, and 20 µl of microtiter of reticulocyte lysate containing [35S]cysteine-labeled apocytochrome c. The reaction was started by adding freshly prepared sodium dithionite to a final concentration of 1 mM/ml and incubating for 10 min at 25°C. The reaction was cooled to 0°C and lysed by adding 1 ml of buffer containing 1% (w/v) Triton X-100, 0.3% NaCl, and 10 mM Mops/KOH, pH 7.2. Samples were immunoprecipitated using cytochrome c-specific antiserum. The amounts of apocytochrome c and holocytochrome c were determined by liquid scintillation counting.

Import of Mitochondrial c-type Cytochromes

Mitochondria were incubated for 15 min at 25 °C in a mixture containing 1% Triton X-100, 0.3 M NaCl, 10 mM Tris/HCl, pH 7.4, and samples were immunoprecipitated using cytochrome c-specific antibodies. Depending on whether the processing state of the imported pScl-c and pLc-c was to be determined or whether the amount of apo species converted to the heme-containing species was to be quantified, immunoprecipitates were analyzed by SDS-PAGE or were prepared for analysis by reverse-phase HPLC as described before (Nicholson et al., 1987). Levels of holocytochrome c, formed were quantified as described by Nicholson et al. (1989).

Miscellaneous

Published procedures were used for the following: preparation of holocytochrome c and apocytochrome c and production of specific antibodies in rabbits (Hennig and Neupert, 1983); protein determination (Bradford, 1976); subfractionation of mitochondria by treatment with digitonin following import analysis (Hartl et al., 1986); measurement of adenylate kinase (Schmidt et al., 1984) and fumarase (Kanarek and Hill, 1964); immunoprecipitation and preparation of samples for SDS-PAGE (Nicholson et al., 1987); SDS-PAGE (Laemmli, 1970); radioactivity determination, fluorography (Nicholson et al., 1987); protease pretreatment of mitochondria (Nicholson et al., 1988; Stuart et al., 1990); treatment of reticulocyte lysates with apyrase (Pfanner et al., 1987a); preparation of and competition studies with water-soluble porin (Pfanner and Neupert, 1987; Pfanner et al., 1988; Stuart et al., 1990); overexpression, isolation, and competition studies with the b-dihydrofolate reductase fusion protein (Rassow et al., 1988).

RESULTS

Import of pLc-c into Mitochondria—pLc-c is a fusion protein comprised of the entire cytochrome c, a protein comprised of the same mitochondrial matrix targeting domain of the cytochrome c1 presequence, which is removed by the matrix processing peptidase (MPP), which is represented by a bar. Hatched areas indicate sequences of cytochrome c1 origin; dotted areas are of apocytochrome origin. Amino acids 1-27 represent the first portion of the cytochrome c1 sequence, which is removed by the matrix processing peptidase (MPP), which cleaves between amino acids 27 and 28. Amino acids 28-70 represent the second part of the cytochrome c1 sequence, and the site of the second processing peptide (2nd PP) is indicated. Amino acids 71-73 are the first 3 amino acids of the mature cytochrome c1 sequence. Amino acids 7-107 represent the apocytochrome c part of the fusion protein. Cysteine sulfhydryl residues to which the heme becomes attached are also indicated (—SH). The region of fusion is enlarged, and the details of the coding sequence, amino acid sequence, and amino acid numbering are indicated. The nucleotide introduced by site-directed mutagenesis is indicated by a box.

When import of pLc-c was performed in the presence of EDTA and o-phenanthroline (o-phe) (tracks 1), 2.5 mM MgCl2 (tracks 2-5), sodium dithionite (1 mg/ml) (tracks 4 and 5), or a mixture of antimycin A (AA), oligomycin (Oligo) and valinomycin (Val) at final concentrations of 8, 20, and 0.5 μM, respectively. Following protease treatment, samples were divided in half, and LeC was immunoprecipitated. A, one set of samples was analyzed by SDS-PAGE and fluorography. The bands corresponding to LeC were detected in the resulting film. B, the other set of samples was dissociated in urea, digested with trypsin, and subjected to reverse-phase HPLC. The amounts of apo- and holo-LeC were quantified by collecting the corresponding peptides and determining their radioactivity. Results are given as the percentage of the total mitochondrial-associated cytochrome c present as either apo-pLc-c or holo-LeC.
protein concentration of 5 mg/ml. Afterward, samples were diluted from one set of samples and analyzed either by SDS-PAGE, supernatant fractions were divided in half. Lc,-c was immunorecognition and were used for the determination of marker enzyme activities, both c and e pathways were active (i.e., +ΔΨ, +reductant), the only protease-resistant species to accumulate in the mitochondria was iLc,-c (Fig. 2A, lane 3). Formation of holo-Lc,-c (import +ΔΨ, +NADH) was almost as efficient as that of holo-pLc,-c formation (import +ΔΨ, +NADH) (Fig. 2B, tracks 5 versus 4), thus suggesting that holo-Lc,-c formation was entirely independent of ΔΨ, a situation similar to the formation of the authentic cytochrome c. Furthermore, it would appear that the holo-Lc,-c may be accumulated in the mitochondria either as a precursor or intermediate size Lc,-c depending on whether a membrane potential was present or not. We conclude, therefore, that both the matrix and cytochrome c-targeting information in the pLc,-c hybrid protein are functional and thus can be selectively imported into mitochondria along either pathway.

Although the heme group was covalently attached to the intermediate size Lc,-c fusion protein (import +ΔΨ, +reductant), further processing to mature size Lc,-c (mLc,-c) was never observed. We have demonstrated recently that the second processing event of the apocytochrome c presquence, which takes place at the outer surface of the inner membrane, occurs only after the covalent heme attachment to cytochrome c, has taken place and not before (Nicholson et al., 1989). This heme addition reaction is catalyzed by cytochrome c, heme lyase. Cytochrome c, heme lyase-catalyzed holo formation of the iLc,-c accumulated in the mitochondria did not take place (results below; see Fig. 8); consequently, the second processing event also could not occur, and formation of mLc,-c was not observed. The lack of the second processing event may also suggest that the processing enzyme involved may require some additional information encoded for by the mature part of the cytochrome c protein. This would be of course lacking in the case of pLc,-c and hence may contribute to the explanation for the lack of observed processing to mature size protein.

Localization of the Apo-iLc,-c and Holo-Lc,-c Species Accumulated in the Mitochondria—The precursor of the Lc,-c fusion protein (pLc,-c) could thus be imported into the mitochondria where it underwent processing to iLc,-c in the presence of a membrane potential and divalent cations. In order to determine the localization of the iLc,-c species, import of [35S]methionine-labeled pLc,-c into the mitochondria was performed at 8°C in the presence of MgCl2 and a membrane potential. Following protease treatment, mitochondria were reisolated and treated with increasing concentrations of digitonin to release sequentially the contents of the intermembrane space and matrix compartments. Accumulation of iLc,-c was released from the mitochondria upon a digitonin treatment that correlated with the release of the matrix marker, fumarase (Fig. 3A). This result thus suggests that at low temperature (8°C), imported iLc,-c can be accumulated in the mitochondrial matrix as a soluble species. On the other hand, ADK activity was halted by the addition of PMSF (2 mM, end concentration). These samples were then treated in a manner identical to those in panel A, which was as follows. Both pellet and supernatant fractions were divided in half, Lc,-c was immunoprecipitated from one set of samples and analyzed either by SDS-PAGE, where the resulting fluorographs were quantified by laser densitometry (panel A and B, A), or the immunoprecipitates were digested with trypsin and subjected to reverse-phase HPLC (panel C, A). The remaining parts of the samples were adjusted to 1% (w/v) Genapol and were used for the determination of marker enzyme activities, adenylate kinase (C) and fumarase (O). For panel A, the activities received in the pellet and supernatant fractions were set to 100% for each digitonin concentration; for panels B and C, activity in the first sample, which received no digitonin, was set as the total 100%.
the other hand, however, if iLc,-c was accumulated in the mitochondria at 25 °C, it was no longer a soluble species but remained membrane bound; only low levels of iLc,-c were released from the membrane fraction into the supernatant upon digitonin treatment (results not shown). Measurement of adenylate kinase and fumarase activities, though, indicated that nearly a complete loss of the soluble components of the intermembrane space and matrix compartments was achieved at the highest levels of digitonin. If the digitonin treatment was followed by proteinase K treatment (Fig. 3B), however, imported iLc,-c remained protected from the added protease only up to the point at which the intermembrane space was opened as a result of the digitonin treatment. This disruption of the outer membrane by the digitonin was monitored by the release of the intermembrane space marker, adenylate kinase.

It may be concluded therefore, that the imported iLc,-c was exposed to the intermembrane space while being anchored to the inner mitochondrial membrane, presumably via the remaining hydrophobic core of the cytochrome c1 presequence. The topology of the imported iLc,-c resembled that of the intermediate size cytochrome c1 accumulated at 25 °C in the absence of reductants (Hartl et al., 1987; Nicholson et al., 1989). When the pLc,-c fusion protein was directed along the cytochrome c route (−ΔΨ, +NADH) the holo-pLc,-c formed was localized in the intermembrane space. Accumulated holo-pLc,-c was only partially soluble; however, when the digitonin treatment was performed, holo-pLc,-c became accessible to added cytochrome c-specific antibodies as soon as the intermembrane space was opened (as judged by the release of adenylate kinase) (Fig. 3C).

**Characteristics of pLc,-c Import along the Cytochrome c1 Route**—Initially we addressed the question of whether the cytochrome c1 receptor and GIP were required for the import of pLc,-c into the mitochondrial matrix. Mitochondria were pretreated with proteinase K (50 μg/ml) and were tested for their ability to import cytochrome c1, pSct,-c, and pLc,-c in comparison with untreated mitochondria (Fig. 4A). Import into mitochondria was controlled by the resistance to externally added proteinase K following the import incubation. The import of cytochrome c1 was abolished in the protease-treated mitochondria, thus demonstrating that the treatment of mitochondria with protease had removed the cytochrome c1 receptor. The accumulation of protease-resistant iLc,-c, like that of mSct,-c, was also independent of the presence of the cytochrome c1 receptor (Stuart et al., 1990).

The question as to whether pLc,-c import into the matrix was dependent on GIP was then addressed. Water-soluble porin was prepared and tested for its ability to compete against pLc,-c import into the matrix at the level of GIP. As shown in Fig. 4B, water-soluble porin was unable to inhibit pLc,-c import at all concentrations used; thus, the membrane insertion activity of the cytochrome c domain renders the function of GIP dispensable.

In summary, pLc,-c may efficiently circumvent receptor proteins and GIP in order to gain access to the mitochondrial matrix. This unique property is apparently bestowed upon this fusion protein because of the membrane-active nature of its apocytochrome c domain.

**Import of pLc,-c Can Occur via Translocation Contact Sites**—Transport of precursors across the two mitochondrial membranes occurs in a single step at contact sites between the outer and inner membranes (Schleyer and Neupert, 1985; Schwaiger et al., 1987). Precursor proteins can be trapped and analyzed in these contact sites (translocation intermediates) by various different approaches such as transport at low temperature or transport after prebinding of precursor pro-

![Fig. 4. Import of pLc,-c into the matrix does not require the cytochrome c1 receptor (MOM19) or GIP. A, pLc,-c was tested for its ability to be imported into protease-pretreated mitochondria. Mitochondria (1 mg of protein/ml) were pretreated with 50 μg of proteinase K per ml as described under "Materials and Methods." Protease-treated mitochondria (+PK) and control mitochondria (−PK) were tested for their ability to import 35S-labeled precytochrome c1, pSct,-c, and pLc,-c in the presence of 2.5 mM MgCl2 for 30 min at 25 °C. Import into the matrix was determined by the formation of protease-resistant intermediate size cytochrome c1 and Lc,-c and mature size pSct,-c. All samples were resolved by SDS-PAGE, and the corresponding bands from the resulting fluorographs are depicted. B, import of pLc,-c does not require GIP. The precursors of pLc,-c, cytochrome c1, and porin, synthesized in reticulocyte lysate in the presence of [35S]methionine, were imported into mitochondria in the presence of varying concentrations of unlabeled water-soluble porin (at concentrations indicated). Following the incubation period, the import of porin was controlled by resistance to 200 μg of proteinase K per ml; import into the matrix was determined by the accumulation of intermediate size cytochrome c1 and Lc,-c. Reisolated mitochondria were lysed with SDS-containing sample buffer, and samples were resolved by SDS-PAGE. Imported porin (●), iC1 (□), and iLc,-c (■) were quantified by subsequent laser densitometry of the fluorographs of the resulting gels.

![Image of Fig. 4](https://example.com/fig4.png)
antiserum or with 40 μl of antiapocytochrome c serum for 30 min at
bound to antibodies can be processed by mitochondria. Two aliquots
imported into the matrix of intact mitochondria. A, (lanes 2) or not (lanes
and were incubated with isolated mitochondria (20 μg of protein)
either in the presence of antimycin A, oligomycin, and valinomycin
was halted by the addition of 1 ml of SME buffer containing 2 mM
was continued for a further 15 min. The reactions were cooled to
7.4). After a clarifying spin, apocytochrome c serum and protein-A-
was added. All immunocomplexes were purified with protein A-
reduction of the antibody-Lc,-c complex was not completely translocated into mi-
ochondria; however, it was processed to the intermediate size form.

It may be thus concluded that pLcl-c can form a translo-
contact site intermediate, spanning both the outer and
inner mitochondrial membranes. We have demonstrated,
however, that pLc,-c, by virtue of its membrane insertion
activity, may bypass receptors and GIP during its import into
the matrix. We then addressed the question of whether this
ability to insert directly into the outer membrane would allow
pLcl-c to gain access to the inner membrane in a manner that
rendered the translocation contact site machinery dispensable.
Recently a fusion protein consisting of the first 187 amino
acids of cytochrome b6 and of the mouse dihydrofolate redu-
tase has been constructed, and it has been demonstrated that
in the presence of methotrexate, import of this protein into
mitochondria was halted at the level of spanning translocation
contact sites (Rassow et al., 1989). Saturation of contact sites
in such a manner affected the import of other authentic mitochondrial proteins. We have used this b6-dihydrofolate
reductase fusion protein, purified following overexpression in
Escherichia coli, to saturate contact sites and to determine
whether the matrix import of pLcl-c is affected in the same
manner as other mitochondrial proteins are. The Fe-S protein
of the b/c1 complex and pLc,-c were synthesized in reticulocyte
lysat and incubated with mitochondria that had accumulated
increasing amounts of b6-dihydrofolate reductase contact site
intermediate. As demonstrated previously, saturation of con-
tact sites with b6-dihydrofolate reductase blocked the import
of Fe-S protein (Rassow et al., 1988). The import of pLc,-c
into the matrix, where it was processed to iLc,-c, however,
was completely unaffected (Fig. 5 B).

To summarize, import of pLcl-c could occur through trans-
location contact sites. In a manner similar to that demonstrated
previously for cytochrome c, antibody-bound pLcl-c could be
accumulated in mitochondria as a translocation contact site intermediate. If, however, import through contact
sites was hindered, as is the case when these sites are jammed
with another precursor protein, b6-dihydrofolate reductase,
import of pLc,-c into the matrix was unaffected. These results
thus suggest that the membrane-active nature of the apocy-
tochrome c passenger protein also enabled circumvention of
these import sites.

Cytocrome c-specific Characteristics of the Import of
pLcl-c into Mitochondria—Nucleoside triphosphates are re-
quired for the import of precursor proteins into mitochondria,
and nonhydrolyzable ATP analogs are not able to fulfill this
NTP requirement (Pfanner and Neupert, 1986; Pfanner
et al., 1987a). It is thus postulated that the hydrolysis of NTPs

SDS-PAGE, and levels of mFe-S and iLcl-c were quantified following
laser densitometry of the resulting fluorographs. Control import
of Fe-S and pLcl-c measured in the absence of b6-dihydrofolate reductase
was set to 100%.
Import of Mitochondrial c-type Cytochromes

is involved in modulating the folding of precursors in the cytosol and thereby maintaining or conferring a conformation on the precursor protein that is more competent for import. In contrast to all precursor proteins studied thus far, however, NTPs are not required throughout the import process of cytochrome c, and thus a specific folded rather than unfolded conformation of apocytochrome c is thought to be required for binding and membrane insertion. It was therefore investigated whether the import of pLcl-c into both the mitochondrial intermembrane space and matrix would be dependent on NTPs or if they shared the characteristic of NTP independence as shown by cytochrome c (Fig. 6).

The precursor proteins pLcl-c, apocytochrome c, and of F,r-ATPase subunit β were synthesized in reticulocyte lysates in the presence of [35S]methionine except for the F,β synthesis in which [35S]methionine was used. Aliquots of pLcl-c and apocytochrome c were pooled separately with an aliquot of F,β lystate, and all reticulocyte lysate mixtures were treated with increasing concentrations of apyrase. Import into a protease-protected location and subsequent processing of pF,β were strongly inhibited by apyrase treatment of the lystate (Fig. 6). As demonstrated previously, holocytochrome c formation was unaffected or even slightly stimulated when reticulocyte lysate containing 35S-labeled apocytochrome c had been treated with apyrase. Unlike F,β, import of pLcl-c into the matrix, where it was processed to iLcl-c, however, was not inhibited by apyrase treatment of the lystate. In fact, a stimulation of import was observed at higher concentrations of apyrase (Fig. 6). A similar result was obtained for the formation of holo-pLcl-c; depletion of NTP levels stimulated the import of the fusion protein along the cytochrome c pathway (Fig. 6). The import pathways of pLcl-c into the matrix thus display the same NTP independence as its import into the intermembrane space along the cytochrome c route.

pLcl-c Cannot Be Reintroduced to the Cytochrome c Pathway When First Imported by the Conservative Sorting Pathway—Cytochrome c, c1 can be accumulated at 25°C in mitochondria as an intermediate size species in the absence of heme. This apo-iC, which is topologically exposed to the intermembrane space while being anchored to the inner membrane, forms a salt-resistant complex with cytochrome c1 heme lyase (Nicholson et al., 1989). Upon addition of a reductant such as sodium dithionite, this apo-iC1 can be chased to its holo form followed by processing to mature size cytochrome c1. The question thus arises as to whether apo-iLcl-c accumulated in the same submembrane location, i.e. exposed to the intermembrane space, could also be chased to its holo counterpart by adding a reductant.

Thus, the precursor to the fusion protein, pLcl-c, was synthesized in a rabbit reticulocyte lysate in the presence of [35S]cysteine. The lysate was incubated with isolated mitochondria either in the presence or absence of sodium dithionite. Following incubation, the samples were treated with protease K to digest the precursor that was not imported into mitochondria, and then the mitochondria were resuspended by centrifugation. The sample that was incubated in the presence of sodium dithionite was kept on ice while the remaining sample was divided into two aliquots and incubated at 25°C for 5 min either in the presence of sodium dithionite or not. All samples were then analyzed by ITPC to allow quantitation of apo- and holo-iLcl-c levels (Fig. 7A).

When import was carried out in the presence of sodium dithionite, almost all of the iLcl-c that was accumulated was holocytochrome c in nature (Fig. 7A, track 1). The intermediate size Le1-c was the only species to accumulate under these import conditions (result not shown). However, in the absence of reductants, no holo formation of the apo-iLcl-c that had been accumulated occurred in the subsequent chase incubation if reducing agents were omitted (Fig. 7A, track 2) or included (Fig. 7A, track 3).

That this failure of the apo-iLcl-c to be chased to holo-iLcl-c did not represent a kinetic problem is shown in Fig. 7B. The kinetics of holocytochrome c1 formation from accumulated apointermediate size c1 have been determined previously (Nicholson et al., 1989). The precursors of cytochrome c1 and Le1-c were synthesized in reticulocyte lysate in the presence of [35S]cysteine. Lysates were incubated (separately) with mitochondria in the absence of reductants at 25°C for 20 min. Following incubation, samples were protease treated, and the mitochondria were resuspended. Chase of the accumulated apointermediate size cytochrome c1 and Le1-c was performed by the addition of sodium dithionite and incubation at 25°C for the time periods indicated. Following the incubations, cytochrome c1 and Le1-c were immunoprecipitated, and HPLC analysis was performed to quantify the levels of apo and holo forms of both cytochrome c1 and Le1-c.

As demonstrated previously, an incubation period of 10 min was optimal for holocytochrome c1 formation (Nicholson et al., 1989). Irrespectively of the time period, however, no efficient holo-iLcl-c formation was observed. It would appear, therefore that once accumulated in mitochondria, apo-iLcl-c cannot be converted to holo-iLcl-c. These results would tend to suggest that the event of covalent heme attachment to pLcl-c observed must occur prior to its entry and/or processing in the mitochondrial matrix.

Cytochrome c Heme Lyase Is Required for Holo-Ncl-c Formation—The covalent attachment of heme to apocytochrome c1 is catalyzed by the enzyme cytochrome c heme lyase and occurs at the outer face of the inner membrane (Nicholson et al., 1989). Cytochrome c1 heme lyase is clearly distinct from cytochrome c heme lyase, the enzyme that catalyzes heme addition to apocytochrome c (Dumont et al., 1987; Nargang et al., 1988). It was then investigated which heme lyase, cytochrome c1 heme lyase or cytochrome c heme lyase, was responsible for the covalent attachment to both the pSc-c and pLcl-c fusion proteins. In order to do this, isolated cyto2 1 mitochondria were employed; these mitochondria are defi-
Import of Mitochondrial c-type Cytochromes

A. Following import, apo-ilc1-c cannot be chased to holo-ilc1-c. A, pLc1-c was synthesized in the presence of [35S]cysteine and was imported into isolated mitochondria either in the presence (track 1) or absence of sodium dithionite (1 mg/ml) (tracks 2 and 3). Following proteinase K treatment, mitochondria were resuspended in SME buffer, and aliquots (equivalent to 50 pg of protein) were then incubated further for 5 min at 25 °C in the presence of 3 μM hemin (tracks 2 and 3) and 1 mg of sodium dithionite per ml (track 3) (final volume 200 μl in SEM buffer). All samples were lysed in Triton X-100-containing buffer, and Lc1-c was immunoprecipitated. Following reverse-phase HPLC, the amounts of protease-resistant apo-ilc1-c and holo-ilc1-c formed upon the chase were quantified by collecting the corresponding peptides and counting their radioactivity. All results are given as the percentage of the total protease-resistant ilc1-c present as either apo-ilc1-c or holo-ilc1-c. The intermediate size Lc1-c was the only species to accumulate under both of these import conditions (results not shown). B. The [35S]cysteine-labeled cytochrome c and pLc1-c were imported separately into mitochondria to their intermediate size forms in two pools equivalent to 50 μg of mitochondrial protein per sample in the presence of 2.5 mM MgCl2 at 25 °C as described under “Materials and Methods.” After treatment with proteinase K, mitochondria were resuspended in SME containing 2 mM PMSF at a protein concentration of 2 mg/ml and portioned into aliquots containing 50 μg of protein each. The imported intermediate size cytochrome c and Lc1-c were then chased for varying periods of time (as indicated) at 25 °C in mixtures (100 μl, final volume in SME buffer) in the presence of 1 mg of sodium dithionite per ml and 3 μM hemin. After the incubation period, cytochrome c and Lc1-c were immunoprecipitated, and the amounts of holocytochrome c and holo-Lc1-c formed were determined as described under “Materials and Methods.”

B. Formation of holocytochrome c1, on the other hand, was dependent on the presence of a ΔΨ (as demonstrated previously) and occurred also in the cyt2-1 mitochondria, thus demonstrating the presence of cytochrome c1, heme lyase activity in these mitochondria (Fig. 8, panel B). Holo Sc1-c formation was observed in the wild-type mitochondria and, as demonstrated previously, was favored by the absence of a ΔΨ. No significant holo-sc1-c formation was detected in the cyt2-1 mitochondria, which is in agreement with previous results (Fig. 8, panel C) (Stuart et al., 1990). The presence or absence of a ΔΨ in wild-type mitochondria had relatively little effect on the holo-Lc1-c formation. More importantly, however, no holo-Lc1-c for-

Fig. 7. Following import, apo-ilc1-c cannot be chased to holo-ilc1-c. A, pLc1-c was synthesized in the presence of [35S]cysteine and was imported into isolated mitochondria either in the presence (track 1) or absence of sodium dithionite (1 mg/ml) (tracks 2 and 3). Following proteinase K treatment, mitochondria were resuspended by centrifugation. Mitochondria from samples 2 and 3 were resuspended in SME buffer, and aliquots (equivalent to 50 ng of protein) were then incubated further for 5 min at 25 °C in the presence of 3 μM hemin (tracks 2 and 3) and 1 mg of sodium dithionite per ml (track 3) (final volume 200 μl in SEM buffer). All samples were lysed in Triton X-100-containing buffer, and Lc1-c was immunoprecipitated. Following reverse-phase HPLC, the amounts of protease-resistant apo-ilc1-c and holo-ilc1-c formed upon the chase were quantified by collecting the corresponding peptides and counting their radioactivity. All results are given as the percentage of the total protease-resistant ilc1-c present as either apo-ilc1-c or holo-ilc1-c. The intermediate size Lc1-c was the only species to accumulate under both of these import conditions (results not shown). B. The [35S]cysteine-labeled cytochrome c and pLc1-c were imported separately into mitochondria to their intermediate size forms in two pools equivalent to 50 μg of mitochondrial protein per sample in the presence of 2.5 mM MgCl2 at 25 °C as described under “Materials and Methods.” After treatment with proteinase K, mitochondria were resuspended by centrifugation and suspended in SME containing 2 mM PMSF at a protein concentration of 2 mg/ml and portioned into aliquots containing 50 μg of protein each. The imported intermediate size cytochrome c and Lc1-c were then chased for varying periods of time (as indicated) at 25 °C in mixtures (100 μl, final volume in SME buffer) in the presence of 1 mg of sodium dithionite per ml and 3 μM hemin. After the incubation period, cytochrome c and Lc1-c were immunoprecipitated, and the amounts of holocytochrome c and holo-Lc1-c formed were determined as described under “Materials and Methods.”

Fig. 8. pLc1-c and pSc1-c are not substrates for cytochrome c1 heme lyase. Apocytochrome c, precytochrome c1, pSc1-c, and pLc1-c were synthesized in reticulocyte lysates in the presence of [35S]cysteine. Mitochondria were isolated freshly from both wild-type and cyt2-1 N. crassa in SME buffer containing 2 mM PMSF. The various lysates (20 μl each) were incubated with either wild-type or cyt2-1 mitochondria (50 μg of protein) in the presence of 2.5 mM MgCl2, 3 μM hemin, sodium dithionite (1 mg/ml), and either in the presence or absence of antimycin A, oligomycin, and valinomycin (as indicated as + or −ΔΨ). Samples were incubated for 20 min at 25 °C and then were lysed with 1 ml or 1% (w/v) Triton X-100-containing buffer. Levels of holocytochrome c (A), holocytochrome c1 (B), holo-Sc1-c (C), and holo-Lc1-c (D) were determined following immunoprecipitation digestion of immunocomplexes with trypsin and subjecting to reverse-phase HPLC, as described under “Materials and Methods.”
mation was observed with the cyt2-1 mitochondria, irrespective of whether the membrane potential was present or not (Fig. 8, panel D).

In summary, cyt2-1 mitochondria contained little or no cytochrome c heme lyase enzyme activity. It can be concluded that in wild-type mitochondria both the pSc1-c and pLc1-c fusion proteins are substrates only for cytochrome c heme lyase, as no holo formation of these species was observed in cyt2-1 mitochondria. Furthermore, these results imply that when imported along a cytochrome c1 route (+ΔΨ), holo-Lc1-c formation can only occur after an interaction with cytochrome c heme lyase has taken place. As it has been demonstrated in Fig. 7, since such an interaction cannot occur subsequent to import of pLc1-c and its processing to iLc1-c in the matrix, it thus must occur prior to it.

**Discussion**

In order to address the question of whether cytochrome c could be functionally sorted to the mitochondrial intermembrane space along a conservative sorting pathway, a fusion protein, termed pLc1-c, was constructed. pLc1-c contains 3-fold targeting information, in addition to a cytochrome c domain. pLc1-c also contains the complete bipartite presequence of the cytochrome c1 precursor. It has been postulated previously that the cytochrome c1 presequence contains the necessary information to target this protein not only to the mitochondria but also to its correct sub mitochondrial location. The proposed import pathways of the pLc1-c are depicted in Fig. 9. The apocytochrome c targeting information in pLc1-c was also found to be functional, and consequently pLc1-c could be imported along a cytochrome c pathway. This resulted in the accumulation of holo-pLc1-c in the intermembrane space of mitochondria. A functional dissection of the ΔΨ-dependent import pathway of the pLc1-c fusion protein revealed that the cytochrome c1 presequence does indeed contain all the necessary information to deliver this protein correctly to its final location, namely, the intermembrane space side of the inner membrane. Upon import along a cytochrome c1 pathway, pLc1-c was initially imported into the matrix from where it became reexported across the inner membrane, resulting in exposure to the intermembrane space. The results presented here not only increase our knowledge and understanding of the import pathways of the c-type cytochromes but they also have many important consequences for the field of mitochondrial protein import in general.

First, it can now be shown directly that the second, hydrophobic domain of the bipartite presequences that many proteins destined for the intermembrane space contain, does indeed act as a reexport signal rather than a stop transfer signal. Bipartite presequences are comprised of a matrix targeting domain that is proteolytically removed in the matrix and a second, rather hydrophobic, domain that is cleaved in the intermembrane space. At low temperature, imported pLc1-c could be accumulated in the mitochondria as a soluble matrix protein, thus arguing strongly against a stop transfer function of the hydrophobic domain (van Loon and Schatz, 1987). Rather, this second domain of the cytochrome c1 presequence appears to be responsible for the reexport of the intermediate size cytochrome c1 out of the matrix to the intermembrane space (Hartl et al., 1986, 1987; Hartl and Neupert, 1990). This hypothesis was supported by the remarkable similarity that exists between the second part of mitochondrial cytochrome c1 presequence and the leader (export) sequences of the bacterial cytochromes c1 and c2. Furthermore, the complete translocation of the hydrophobic domain across both membranes, rather than its retention in the lipid bilayer, as would be suggested by a stop transfer model, supports the previous finding that import of precursor proteins into mitochondria occurs through a hydrophilic environment (Pfanner et al., 1987b). Once pLc1-c was in the matrix, at higher temperatures (25 °C) the second domain of the presequence directed the export of its carrier protein, apocytochrome c, out of the matrix. The apocytochrome c domain thus becomes relocalised across the inner membrane where it was accumulated as an apointermediate size Lc1-c species, exposed to the intermembrane space. This demonstrates that the hydrophobic domain of the presequence alone does indeed contain the necessary information to act as a reexport sequence.

Second, when membrane insertion activity is endogenously present in mitochondrial precursor proteins, the functions of the receptor/GIP system for import are rendered dispensable. pLc1-c did not require the presence of surface receptors of GIP for import into the matrix. Despite the interruption of the two membrane-active domains (i.e. the first part of the cytochrome c, presequence and the apocytochrome c domain) with the second domain of the cytochrome c1 presequence, the presence of the cytochrome c domain as part of the pLc1-c fusion protein endows it with properties of receptor and GIP independence. This second domain of the presequence is very hydrophobic, and therefore it may also become inserted into the outer membrane along with the apocytochrome c and thus does not hinder the matrix targeting domain from being delivered to the vicinity of the ΔΨ-dependent step of matrix import.

Third, ATP-dependent unfolding and passage through the translocation contact site machinery are not essential events in the import process of proteins into the matrix: they can in fact be circumvented. The precursor protein pLc1-c, as demonstrated previously for cytochrome c1, could be accumulated in mitochondria as a translocation contact site intermediate (Schleyer and Neupert, 1985). During import into the matrix of intact mitochondria, pLc1-c could also, however, bypass the translocation contact machinery in an ATP-independent
manner; this is presumably because of the presence of the apocytochrome c domain which enables pLc-c to insert into the outer membrane spontaneously. Upon doing so, pLc-c does not enter translocation contact sites but instead achieves direct access to the inner membrane and presumably crosses the inner membrane at other import sites that remain as yet uncharacterized. The presence of such alternative import sites in the inner mitochondrial membrane have been demonstrated recently in disrupted mitochondria (Hwang et al., 1989).

Fourth, although cytochrome c cannot be sorted functionally along a conserved sorting pathway, following receptor from the matrix, apo-iLc-c can be accumulated in the same submembrane location as apo-iC, (i.e. retranslocated into the intermembrane space but remaining attached to the inner membrane). From this position, however, unlike cytochrome c, apo-pLc-c cannot be converted to its holo counterpart. These results would suggest that cytochrome c heme lyase does not appear to be located at (or pLc-c cannot interact with) the sites of export from the matrix. In the presence of a membrane potential and reductants (i.e. both cytochrome c and heme lyase) the pathway cannot be converted to holocytochrome c.

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