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Import of Apocytochrome *c* into the Mitochondrial Intermembrane Space along a Cytochrome c_1 Sorting Pathway*

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The question of whether cytochrome *c* could be functionally sorted to the mitochondrial intermembrane space along a "conservative sorting" pathway was investigated using a fusion protein termed pLc_1-*c*. pLc_1-*c* contains 3-fold targeting information, namely, the complete bipartite presequence of the cytochrome c_1 precursor joined to the amino terminus of apocytochrome *c*. pLc_1-*c* could be selectively imported into the intermembrane space either directly across the outer membrane along a cytochrome *c* import route or along a cytochrome c_1 route via the matrix. Thus, apocytochrome *c* could be sorted along a conservative sorting pathway; however, following reexport from the matrix, apo-Lc_1-*c* could not be converted to its holo counterpart. Despite the apparent similarity of structure and functional location of the heme lyases and similarity of the heme binding regions in their respective apoproteins, cytochrome *c* heme lyase and cytochrome c_1 heme lyase apparently have different and nonoverlapping substrate specificities.

Mitochondria contain two distinct *c*-type cytochromes, namely, cytochrome *c* and cytochrome c_1. Apart from the similarity in their functions as electron carriers, cytochrome *c* and cytochrome c_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_1, in contrast to cytochrome *c*, uses a much more elaborate and complex pathway to gain access to the mitochondrial intermembrane space.

Cytochrome c_1 is initially synthesized as a precursor protein (precytochrome c_1 or pC_1) 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_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)[1] (Pfaller *et al.*, 1988; Stuart *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_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_1, contrary to the "stop transfer" model, is initially transported into the mitochondrial matrix where it is processed to its intermediate size form (iC_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; Hawlitschek *et al.*, 1988). The remaining carboxyl region of the presequence of cytochrome c_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 iC_1, a process that is catalyzed by cytochrome c_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_1, which results in the production of mature size holocytochrome c_1 (Teintze *et al.*, 1982; Nicholson *et al.*, 1989). The complex assembly pathway used by cytochrome c_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 endosymbiotic 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_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

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[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.

periplasm); this process is termed *conservative sorting* (Hartl and Neupert, 1990).

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; Berkhout *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 holocytochrome *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 holocytochrome *c* (Nargang *et al.*, 1988; Stuart and Neupert, 1990). Although cytochrome *c* heme lyase is distinct from cytochrome c_1 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 c_1, 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 ATP[2] (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, 1989). Cytochrome *c*, on the other hand, is sorted in a nonconservative 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_1-*c* was constructed which contained the entire bipartite presequence of cytochrome c_1 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_1-*c* could be imported selectively along a cytochrome *c* route into the intermembrane space or along a cytochrome c_1 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_1-*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 c_1 heme lyase have nonoverlapping substrate specificities.

[2] D. W. Nicholson, R. A. Stuart, and W. Neupert, in preparation.

MATERIALS AND METHODS

DNA Manipulations and Construction of the pLc_1-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_1-*c* is comprised of the complete presequence of cytochrome c_1 joined to the amino terminus of apocytochrome *c* (see Fig. 1). The construction of the plasmid encoding pLc_1-*c* was performed in a manner identical to that described previously for pSc_1-*c* (Stuart *et al.*, 1990) with the exception that the unique *Pvu*II site introduced by site-directed mutagenesis in the cytochrome c_1 cDNA sequence was achieved by changing a cytosine residue for an adenine at a position corresponding to codon 73. pLc_1-*c* is a fusion protein consisting of the entire presequence of cytochrome c_1 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 in reticulocyte 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

N. crassa (wild-type 74A) was grown for 15 h at 25 °C with vigorous aeration and bright illumination as described previously (Hennig and Neupert, 1983). The *N. crassa cyt2-1* 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, 1985) in SME buffer (250 mM sucrose, 2 mM EDTA, 10 mM Mops/KOH, pH 7.2) plus 2 mM PMSF. PMSF was omitted from the SME 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 c_1, the Fe-S protein from the b/c_1 complex, F_1-ATPase subunit β ($F_1\beta$), porin, pSc_1-*c*, and pLc_1-*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; Kleene *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-[^{35}S]cysteine or L-[^{35}S]methionine (Amersham Corp.) as indicated, as described previously (Nicholson *et al.*, 1987). The postribosomal 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 (final volume, 200 microliters) containing SME buffer, 3 μM hemin, and 20 microliters of reticulocyte lysate containing [^{35}S]cysteine-labeled apocytochrome *c*. The reaction was started by adding freshly prepared sodium dithionite to a final concentration of 1 mg/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 M NaCl, and 10 mM Mops/KOH, pH 7.2. Samples were immunoprecipitated using cytochrome *c*-specific antiserum. The amounts of apocytochrome *c* and holocy-

tochrome *c* formed were determined by subsequent HPLC analysis, exactly as described by Nicholson *et al.* (1988).

Import of F_1-ATPase Subunit β, Cytochrome c_1, pSc_1-c, and pLc_1-c—Unless otherwise indicated, mitochondria (50 microliters of protein) were incubated for 15 min at 25 °C in a mixture (final volume, 100 microliters) containing 3% (w/v) bovine serum albumin, 70 mM KCl, 220 mM sucrose, 10 mM Mops/KOH, pH 7.2, and 15 microliters of reticulocyte lysate containing ^{35}S-labeled precursor proteins (Stuart *et al.*, 1990). The import of $pF_1\beta$ was routinely carried out in the presence of 16 mM potassium ascorbate, pH 7, and 0.4 mM *N,N,N′,N′*-tetramethylphenylenediamine. (Note that when more than one sample required the same treatment, pools were prepared which were the appropriate multiples of the individual import mixture but were otherwise treated the same.) Following the import incubation samples were cooled to 0 °C and treated with 20 μg of proteinase K per ml for 30 min at 0 °C. The samples were diluted with SME buffer containing 2 mM PMSF, mitochondria were reisolated by centrifugation at 17,400 × *g* for 12 min, lysed in SDS-containing sample buffer, and resolved by SDS-polyacrylamide electrophoresis. For analysis of pSc_1-c and pLc_1-c import, however, following reisolation mitochondria were lysed in 1 ml of 1% (w/v) 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 pSc_1-c and pLc_1-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.*, 1988; Stuart *et al.*, 1990). Levels of holocytochrome c_1 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 (Pfaller and Neupert, 1987; Pfaller *et al.*, 1988; Stuart *et al.*, 1990); overexpression, isolation, and competition studies with the b_2-dihydrofolate reductase fusion protein (Rassow *et al.*, 1989).

RESULTS

Import of pLc_1-c into Mitochondria—pLc_1-c is a fusion protein comprised of the entire cytochrome c_1 presequence joined to the amino terminus of apocytochrome *c* (Fig. 1). Consequently, like pSc_1-c, a related fusion protein that we have reported on recently (Stuart *et al.*, 1990), pLc_1-c also contains different sets of targeting information and, at the same time, membrane insertion activity. However, in addition to the matrix targeting domain of the cytochrome c_1 presequence which pSc_1-c contains, pLc_1-c also contains the second, hydrophobic, domain of the c_1 presequence. This second domain of the presequence is thought to direct the export of the intermediate size cytochrome c_1 (iC_1) from the mitochondrial matrix across the inner membrane to its final location exposed to the intermembrane space (Hartl *et al.*, 1987).

The precursor of the Lc_1-c fusion protein (pLc_1-c) was synthesized in the presence of [^{35}S]cysteine in a rabbit reticulocyte lysate and was imported to a protease-resistant location in the mitochondria at 25 °C under various incubation conditions (Fig. 2). Following protease treatment, samples were divided and analyzed either by HPLC analysis, to allow quantitation of apo- and holocytochrome *c*, or by SDS-PAGE and fluorography, in order to determine the levels of pLc_1-c and processed intermediate size Lc_1-c (iLc_1-c).

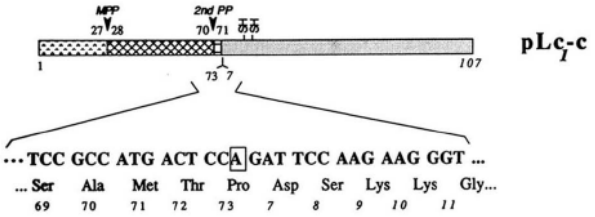

FIG. 1. **Construction of pLc_1-c, a fusion protein between cytochrome c_1 and apocytochrome *c*.** Details of the construction of the pLc_1-c fusion protein are described under "Materials and Methods." pLc_1-c is a fusion protein consisting of the entire presequence of cytochrome c_1 plus 3 amino acids 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. This polypeptide is represented by a *bar*. *Hatched areas* indicate sequences of cytochrome c_1 origin; *dotted areas* are of apocytochrome origin. Amino acids 1–27 represent the first portion of the cytochrome c_1 presequence 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 c_1 presequence, and the site of the second processing peptidase (*2nd PP*) is indicated. Amino acids 71–73 are the first 3 amino acids of the mature cytochrome c_1 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*.

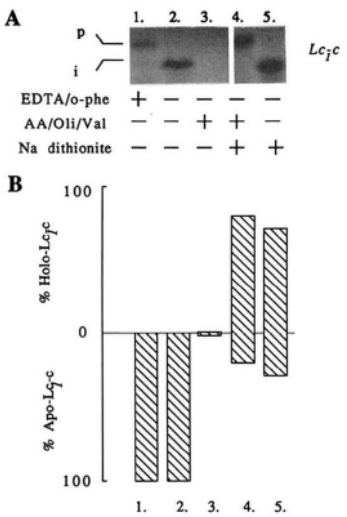

FIG. 2. **Import of pLc_1-c into mitochondria.** The precursor of Lc_1-c synthesized in the presence of [^{35}S]cysteine was imported into isolated mitochondria (100 μg of protein) at 25 °C in the presence of 5 mM EDTA plus 0.2 mM *o*-phenanthroline (*o-phe*) (*track 1*), 2.5 mM $MgCl_2$ (*tracks 2–5*), sodium dithionite (1 mg/ml) (*tracks 4* and *5*), or a mixture of antimycin A (*AA*), oligomycin (*Oli*) and valinomycin (*Val*) at final concentrations of 8, 20, and 0.5 μM, respectively. Following protease treatment, samples were divided in half, and Lc_1-c was immunoprecipitated. *A*, one set of samples was analyzed by SDS-PAGE and fluorography. The *bands* corresponding to Lc_1-c in the resulting film are shown. *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-Lc_1-c were quantified by collecting the corresponding peptides and determining their radioactivity. Results are given as the percentage of the total mitochondria-associated cytochrome *c* present as either apo-pLc_1-c or holo-Lc_1-c.

When import of pLc_1-c was performed in the presence of EDTA and *o*-phenanthroline (Fig. 2*A*, *lane 1*), accumulation of protease-resistant pLc_1-c was observed. In the presence of $MgCl_2$, however, pLc_1-c was imported and processed to iLc_1-c (Fig. 2*A*, *lane 2*). Import of pLc_1-c into mitochondria did not occur in the absence of a membrane potential ($\Delta\Psi$) (Fig. 2*A*,

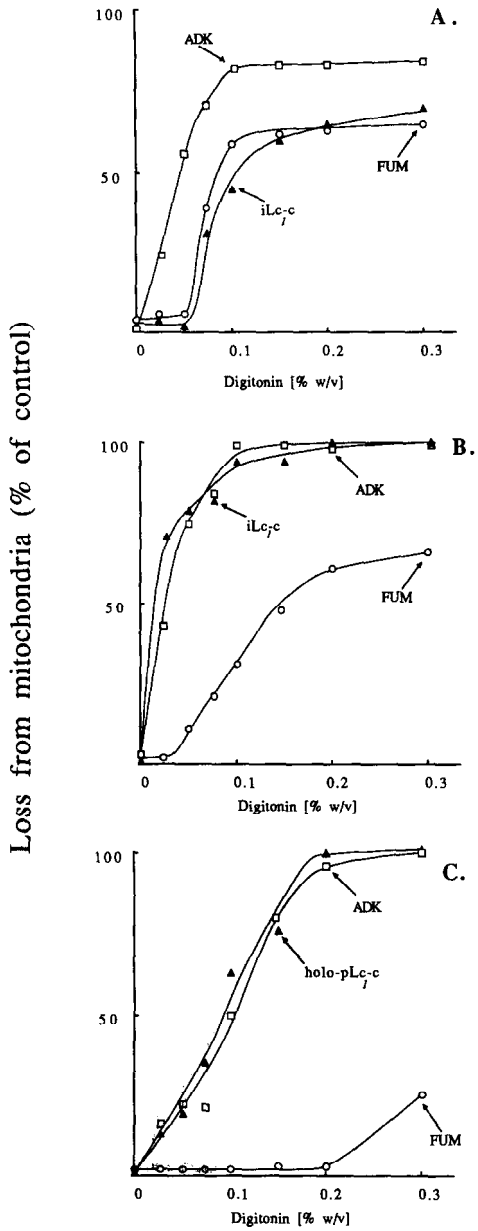

FIG. 3. **Submitochondrial localization of the imported Lc_1-*c* species by digitonin treatment of mitochondria.** The [^{35}S]methionine-labeled pLc_1-*c* was imported into mitochondria (675 μg of protein, 2.25 ml, final volume) in the presence of 2.5 mM $MgCl_2$ at either 8 °C (*panel A*) or at 25 °C (*panel B*). In a further reaction mixture, [^{35}S]cysteine-labeled pLc_1-*c* was imported into mitochondria in the presence of antimycin A, oligomycin, and valinomycin (see Fig. 2 legend) and sodium dithionite (1 mg/ml) (*panel C*). Following proteinase K treatment, mitochondria were isolated and resuspended in SME buffer containing 100 mM KCl (SMEK) and divided into eight 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. Afterward, samples were diluted 20-fold with SMEK and were separated into pellet and supernatant fractions by centrifugation for 10 min at 27,000 × *g* (Beckman, JA20 rotor). In the case of iLc_1-*c* accumulation at 25 °C (*panel B*) and holo-pLc_1-*c* formation (*panel C*), prior to centrifugation samples were treated with 25 or 60 μg proteinase K per ml, respectively, at 0 °C for 30 min. Protease 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_1-*c* was immunoprecipitated from one set of samples and analyzed either by SDS-PAGE, where the resulting fluorographs were quantified by laser densitometry (*panels A* and *B*, ▲), or the immunoprecipitates were digested with trypsin and subjected to reverse-phase HPLC (*panel C*, ▲). 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 (□) and fumarase (○). 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 loss from the mitochondria is plotted. *ADK*, adenylate kinase; *FUM*, fumarase.

lane 3). However, when a reductant such as sodium dithionite was present to power holocytochrome *c* formation, import along a cytochrome *c* pathway occurred (Fig. 2*A*, *lane 4*). Under these conditions, heme-containing pLc_1-*c* was accumulated in a protease-resistant location (Fig. 2, *A* and *B*, *lane 4* and *track 4*). Formation of holo-Lc_1-*c* did not occur in the absence of a reductant in the incubation reaction (Fig. 2*B*, *tracks 4* and *5 versus tracks 1, 2,* and *3*). When both the c_1 and *c* pathways were active (*i.e.* +$\Delta\Psi$, +reductant), the only protease-resistant species to accumulate in the mitochondria was iLc_1-*c* (Fig. 2*A*, *lane 5*). Formation of holo-iLc_1-*c* (import +$\Delta\Psi$, +NADH) was almost as efficient as that of holo-pLc_1-*c* formation (import −$\Delta\Psi$, +NADH) (Fig. 2*B*, *tracks 5 versus 4*), thus suggesting that holo-Lc_1-*c* formation was entirely independent of $\Delta\Psi$, a situation similar to the formation of the authentic cytochrome *c*. Furthermore, it would appear that the holo-Lc_1-*c* may be accumulated in the mitochondria either as a precursor or intermediate size Lc_1-*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_1-*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_1-*c* fusion protein (import +$\Delta\Psi$, +reductant), further processing to mature size Lc_1-*c* (mLc_1-*c*) was never observed. We have demonstrated recently that the second processing event of the apocytochrome *c* presequence, which takes place at the outer surface of the inner membrane, occurs only after the covalent heme attachment to cytochrome c_1 has taken place and not before (Nicholson *et al.*, 1989). This heme addition reaction is catalyzed by cytochrome c_1 heme lyase. Cytochrome c_1 heme lyase-catalyzed holo formation of the iLc_1-*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_1-*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_1 protein. This would be of course lacking in the case of pLc_1-*c* and hence may contribute to the explanation for the lack of observed processing to mature size protein.

Localization of the Apo-iLc_1-c and Holo-Lc_1-c Species Accumulated in the Mitochondria—The precursor of the Lc_1-*c* fusion protein (pLc_1-*c*) could thus be imported into the mitochondria where it underwent processing to iLc_1-*c* in the presence of a membrane potential and divalent cations. In order to determine the localization of the iLc_1-*c* species, import of [^{35}S]methionine-labeled pLc_1-*c* into the mitochondria was performed at 8 °C in the presence of $MgCl_2$ 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. Accumulated iLc_1-*c* was released from the mitochondria upon a digitonin treatment that correlated with the release of the matrix marker, fumarase (Fig. 3*A*). This result thus suggests that at low temperature (8 °C), imported iLc_1-*c* can be accumulated in the mitochondrial matrix as a soluble species. On

the other hand, however, if iLc_1-*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_1-*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. 3*B*), however, imported iLc_1-*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_1-*c* was exposed to the intermembrane space while being anchored to the inner mitochondrial membrane, presumably via the remaining hydrophobic core of the cytochrome c_1 presequence. The topology of the imported iLc_1-*c* resembled that of the intermediate size cytochrome c_1 accumulated at 25 °C in the absence of reductants (Hartl *et al.*, 1987; Nicholson *et al.*, 1989). When the pLc_1-*c* fusion protein was directed along the cytochrome *c* route ($-\Delta\Psi$, +NADH) the holo-pLc_1-*c* formed was localized in the intermembrane space. Accumulated holo-pLc_1-*c* was only partially soluble; however, when the digitonin treatment was performed, holo-pLc_1-*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. 3*C*).

Characteristics of pLc_1-c Import along the Cytochrome c_1 Route—Initially we addressed the question of whether the cytochrome c_1 receptor and GIP were required for the import of pLc_1-*c* into the mitochondrial matrix. Mitochondria were pretreated with proteinase K (50 μg/ml) and were tested for their ability to import cytochrome c_1, pSc_1-*c*, and pLc_1-*c* in comparison with untreated mitochondria (Fig. 4*A*). Import into mitochondria was controlled by the resistance to externally added proteinase K following the import incubation. The import of cytochrome c_1 was abolished in the protease-treated mitochondria, thus demonstrating that the treatment of mitochondria with protease had removed the cytochrome c_1 receptor. The accumulation of protease-resistant iLc_1-*c*, like that of mSc_1-*c*, was also independent of the presence of the cytochrome c_1 receptor (Stuart *et al.*, 1990).

The question as to whether pLc_1-*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_1-*c* import into the matrix at the level of GIP. As shown in Fig. 4*B*, water-soluble porin was unable to inhibit pLc_1-*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_1-*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_1-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 proteins to specific antibodies. These partially transported precursors are accessible to externally added proteases yet have undergone processing by the matrix-located processing peptidase. The latter approach, namely, of prebinding antibodies to the pLc_1-*c* precursor protein, was performed in order to investigate whether the import of pLc_1-*c* into the matrix could occur via contact sites.

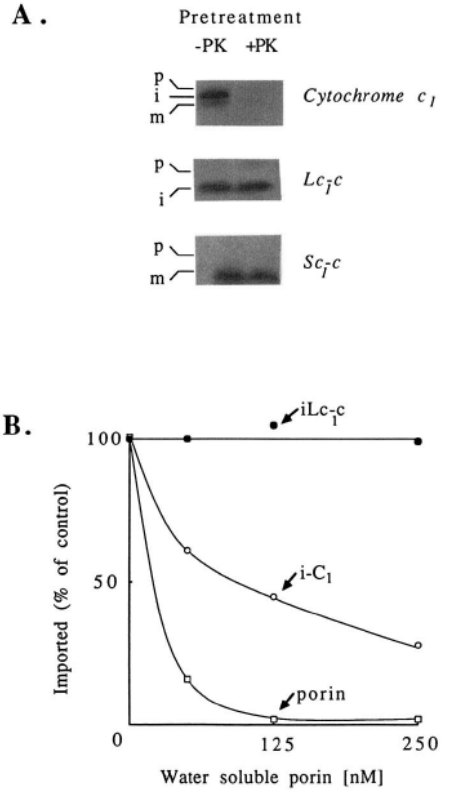

FIG. 4. **Import of pLc_1-*c* into the matrix does not require the cytochrome c_1 receptor (MOM19) or GIP.** *A*, pLc_1-*c* was tested for its ability to be imported into protease-pretreated mitochondria. Mitochondria (1 mg of protein/ml) were pretreated with 50 μg of proteinase K per ml as described under "Materials and Methods." Protease-treated mitochondria (+*PK*) and control mitochondria (−*PK*) were tested for their ability to import ^{35}S-labeled precytochrome c_1, pSc_1-*c*, and pLc_1-*c* in the presence of 2.5 mM $MgCl_2$ for 20 min at 25 °C. Import into the matrix was determined by the formation of protease-resistant intermediate size cytochrome c_1 and Lc_1-*c* and mature size pSc_1-*c*. All samples were resolved by SDS-PAGE, and the corresponding bands from the resulting fluorographs are depicted. *B*, import of pLc_1-*c* does not require GIP. The precursors of pLc_1-*c*, cytochrome c_1, and porin, synthesized in reticulocyte lysate in the presence of [^{35}S]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 c_1 and Lc_1-*c*. Reisolated mitochondria were lysed with SDS-containing sample buffer, and samples were resolved by SDS-PAGE. Imported porin (□), iC_1 (○), and iLc_1-*c* (■) were quantified by subsequent laser densitometry of the fluorographs of the resulting gels.

[^{35}S]Methionine-labeled pLc_1-*c* was synthesized in reticulocyte lysate and was prebound to anti-apocytochrome *c* antibodies by incubating the reticulocyte lysate with antisera against apocytochrome *c*. In the case of control samples, the reticulocyte lysate was incubated with control serum (preimmune serum). Isolated mitochondria were then added to the reticulocyte lysates, and import was performed under different conditions (Fig. 5). From the mitochondria of the incubations that had received control serum, Lc_1-*c* was immunoprecipi-

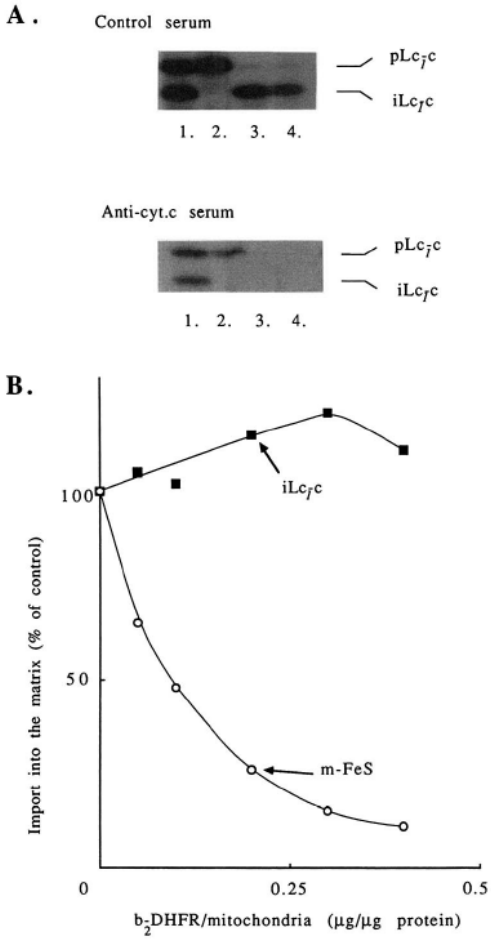

FIG. 5. **pLc$_1$-c can bypass translocation contact sites to be imported into the matrix of intact mitochondria.** *A*, pLc$_1$-c bound to antibodies can be processed by mitochondria. Two aliquots (each 350 μl) of reticulocyte lysate (50 μl) containing [^{35}S]methionine-labeled pLc$_1$-c, supplemented with bovine serum albumin buffer (see "Materials and Methods"), were incubated with either 40 μl of control antiserum or with 40 μl of antiapocytochrome *c* serum for 30 min at 25 °C. The mixes were then divided into four aliquots (each of 90 μl) and were incubated with isolated mitochondria (20 μg of protein) either in the presence of antimycin A, oligomycin, and valinomycin (*lanes 2*) or not (*lanes 1, 3*, and *4*) and incubated for 20 min at 25 °C. Samples were then cooled to 0 °C, and samples 3 and 4 were treated with 30 μg of proteinase K per ml for 30 min at 0 °C. Protease activity was halted by the addition of 1 ml of SME buffer containing 2 mM PMSF, and the mitochondria were reisolated by centrifugation. Mitochondria of samples 1–3 were lysed in 1 ml of 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris/HCl (pH 7.4), and those of reaction 4 were lysed in 50 μl of 1% (w/v) SDS, 10 mM Tris/HCl (pH 8.0) followed by boiling at 95 °C for 5 min and then were also diluted with 1 ml of 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris/HCl (pH 7.4). After a clarifying spin, anticytochrome *c* serum and protein A-Sepharose were added to the control samples whereas protein A-Sepharose alone was added to the other set of samples. Immunoprecipitation was performed, and the immunocomplexes were dissociated in a SDS-containing buffer and were analyzed by SDS-PAGE. The corresponding fluorographs from the resulting gels are depicted. *B*, inhibition of the import of Fe-S and pLc$_1$-c by b_2-dihydrofolate reductase (*DHFR*) translocation intermediate. Mitochondria (10 μg of protein) were incubated with increasing amounts of b_2 dihydrofolate reductase (in 40 μl of 8 M urea) in the presence of 100 nM methotrexate, 16 mM potassium ascorbate (pH 7.2), and 0.4 mM *N,N,N′,N′*-tetramethylphenylenediamine in an end volume of 700 μl of bovine serum albumin buffer for 20 min at 25 °C. Then 5 μl of reticulocyte lysate containing either radiolabeled precursor to the Fe-S protein of the b/c_1 complex or pLc$_1$-c was added, and the incubation was continued for a further 15 min. The reactions were cooled to 0 °C, and the mitochondria were reisolated by centrifugation and lysed in SDS-containing sample buffer. Samples were resolved by SDS-PAGE, and levels of mFe-S and iLc$_1$-c were quantified following laser densitometry of the resulting fluorographs. Control import of Fe-S and pLc$_1$-c measured in the absence of b_2-dihydrofolate reductase was set to 100%.

tated by adding cytochrome *c* antiserum. For immunoprecipitation from mitochondria of those incubations that had received apocytochrome *c* antisera, no further antisera were added. All immunocomplexes were purified with protein A-Sepharose and analyzed by SDS-PAGE (Fig. 5*A*). Processing of pLc$_1$-c to iLc$_1$-c was dependent on a membrane potential, and it occurred irrespective of the presence or absence of prebound apocytochrome *c*-specific antibodies (Fig. 5*A*, *lanes 1* and *2*). The antibody-prebound iLc$_1$-c, however, remained sensitive to added protease unlike Lc$_1$-c, which was imported in the presence of control serum. The integrity of the mitochondrial outer membrane was not adversely affected by these incubation treatments (results not shown). Thus, the antibody-Lc$_1$-c complex was not completely translocated into mitochondria; however, it was processed to the intermediate size form.

It may be thus concluded that pLc$_1$-c can form a translocation contact site intermediate, spanning both the outer and inner mitochondrial membranes. We have demonstrated, however, that pLc$_1$-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 pLc$_1$-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 167 amino acids of cytochrome b_2 and of the mouse dihydrofolate reductase 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 b_2-dihydrofolate reductase fusion protein, purified following overexpression in *Escherichia coli*, to saturate contact sites and to determine whether the matrix import of pLc$_1$-c is affected in the same manner as other mitochondrial proteins are. The Fe-S protein of the b/c_1 complex and pLc$_1$-c were synthesized in reticulocyte lysate and incubated with mitochondria that had accumulated increasing amounts of b_2-dihydrofolate reductase contact site intermediate. As demonstrated previously, saturation of contact sites with b_2-dihydrofolate reductase blocked the import of Fe-S protein (Rassow *et al.*, 1989). The import of pLc$_1$-c into the matrix, where it was processed to iLc$_1$-c, however, was completely unaffected (Fig. 5*B*).

To summarize, import of pLc$_1$-c could occur through translocation contact sites. In a manner similar to that demonstrated previously for cytochrome c_1, antibody-bound pLc$_1$-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, b_2-dihydrofolate reductase, import of pLc$_1$-c into the matrix was unaffected. These results thus suggest that the membrane-active nature of the apocytochrome *c* passenger protein also enabled circumvention of these import sites.

Cytochrome c-specific Characteristics of the Import of pLc$_1$-c into Mitochondria—Nucleoside triphosphates are required 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

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*,[2] 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 pLc_1-*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 pLc_1-*c*, apocytochrome *c*, and of F_1F_0-ATPase subunit β were synthesized in reticulocyte lysates in the presence of [^{35}S]cysteine except for the p$F_1\beta$ synthesis in which [^{35}S]methionine was used. Aliquots of pLc_1-*c* and apocytochrome *c* were pooled separately with an aliquot of p$F_1\beta$ lysate, and all reticulocyte lysate mixtures were treated with increasing concentrations of apyrase. Import into a protease-protected location and subsequent processing of p$F_1\beta$ were strongly inhibited by apyrase treatment of the lysate (Fig. 6). As demonstrated previously, holocytochrome *c* formation was unaffected or even slightly stimulated when reticulocyte lysate containing ^{35}S-labeled apocytochrome *c* had been treated with apyrase.[2] Unlike $F_1\beta$, import of pLc_1-*c* into the matrix, where it was processed to iLc_1-*c*, however, was not inhibited by apyrase treatment of the lysate. 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-pLc_1-*c*; depletion of NTP levels stimulated the import of the fusion protein along the cytochrome *c* pathway (Fig. 6). The import pathways of pLc_1-*c* into the matrix thus display the same NTP independence as its import into the intermembrane space along the cytochrome *c* route.

pLc_1-c Cannot Be Reintroduced to the Cytochrome c Pathway When First Imported by the Conservative Sorting Pathway—Cytochrome c_1 can be accumulated at 25 °C in mitochondria as an intermediate size species in the absence of heme. This apo-iC_1, which is topologically exposed to the intermembrane space while being anchored to the inner membrane, forms a salt-resistant complex with cytochrome c_1 heme lyase (Nicholson *et al.*, 1989). Upon addition of a reductant such as sodium dithionite, this apo-iC_1 can be chased to its holo form followed by processing to mature size cytochrome c_1. The question thus arises as to whether apo-iLc_1-*c* accumulated in the same submitochondrial location, *i.e.* exposed to the intermembrane space, could also be chased to its holo counterpart by adding a reductant.

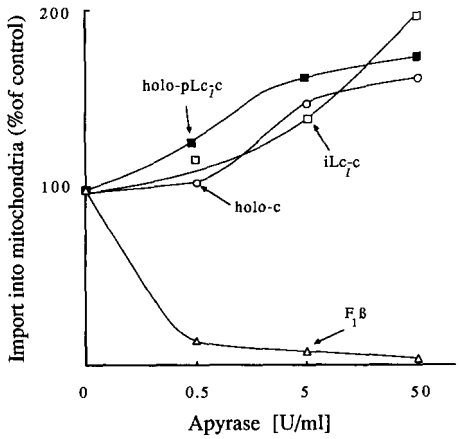

FIG. 6. **Depletion of ATP levels does not interfere with import of pLc_1-*c* along both the cytochrome c_1 and cytochrome *c* pathways.** Reticulocyte lysate containing [^{35}S]cysteine-labeled apocytochrome *c*, pLc_1-*c*, and [^{35}S]methionine-labeled F_1-ATPase subunit β were treated with apyrase as described under "Materials and Methods," and import into isolated mitochondria was performed as described in Fig. 2. The amounts of holocytochrome *c* (○) and holo-pLc_1-*c* (■) formed and the amounts of protease-resistant m$F_1\beta$ (▲) and iLc_1-*c* (□) accumulated in mitochondria are expressed as a percentage of the control that was not treated with apyrase.

Thus, the precursor to the fusion protein, pLc_1-*c*, was synthesized in a rabbit reticulocyte lysate in the presence of [^{35}S]cysteine. The lysate was incubated with isolated mitochondria either in the presence or absence of sodium dithionite. Following incubation, the samples were treated with proteinase K to digest the precursor that was not imported into mitochondria, and then the mitochondria were reisolated 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 HPLC to allow quantitation of apo- and holo-iLc_1-*c* levels (Fig. 7*A*).

When import was carried out in the presence of sodium dithionite, almost all of the iLc_1-*c* that was accumulated was holocytochrome *c* in nature (Fig. 7*A*, *track 1*). The intermediate size Lc_1-*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-iLc_1-*c* that had been accumulated occurred in the subsequent chase incubation if reducing agents were omitted (Fig. 7*A*, *track 2*) or included (Fig. 7*A*, *track 3*).

That this failure of the apo-iLc_1-*c* to be chased to holo-iLc_1-*c* did not represent a kinetic problem is shown in Fig. 7*B*. The kinetics of holocytochrome c_1 formation from accumulated apointermediate size c_1 have been determined previously (Nicholson *et al.*, 1989). The precursors of cytochrome c_1 and Lc_1-*c* were synthesized in reticulocyte lysate in the presence of [^{35}S]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 reisolated. Chase of the accumulated apointermediate size cytochrome c_1 and Lc_1-*c* was performed by the addition of sodium dithionite and incubation at 25 °C for the time periods indicated. Following the incubations, cytochrome c_1 and Lc_1-*c* were immunoprecipitated, and HPLC analysis was performed to quantify the levels of apo and holo forms of both cytochrome c_1 and Lc_1-*c*.

As demonstrated previously, an incubation period of 10 min was optimal for holocytochrome c_1 formation (Nicholson *et al.*, 1989). Irrespective of the time period, however, no efficient holo-iLc_1-*c* formation was observed. It would appear, therefore that once accumulated in mitochondria, apo-iLc_1-*c* cannot be converted to holo-iLc_1-*c*. These results would tend to suggest that the event of covalent heme attachment to pLc_1-*c* observed must occur prior to its entry and/or processing in the mitochondrial matrix.

Cytochrome c Heme Lyase Is Required for Holo-Lc_1-c Formation—The covalent attachment of heme to apocytochrome c_1 is catalyzed by the enzyme cytochrome c_1 heme lyase and occurs at the outer face of the inner membrane (Nicholson *et al.*, 1989). Cytochrome c_1 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 c_1 heme lyase or cytochrome *c* heme lyase, was responsible for the covalent attachment to both the pSc_1-*c* and pLc_1-*c* fusion proteins. In order to do this, isolated *cyt2-1* mitochondria were employed; these mitochondria are defi-

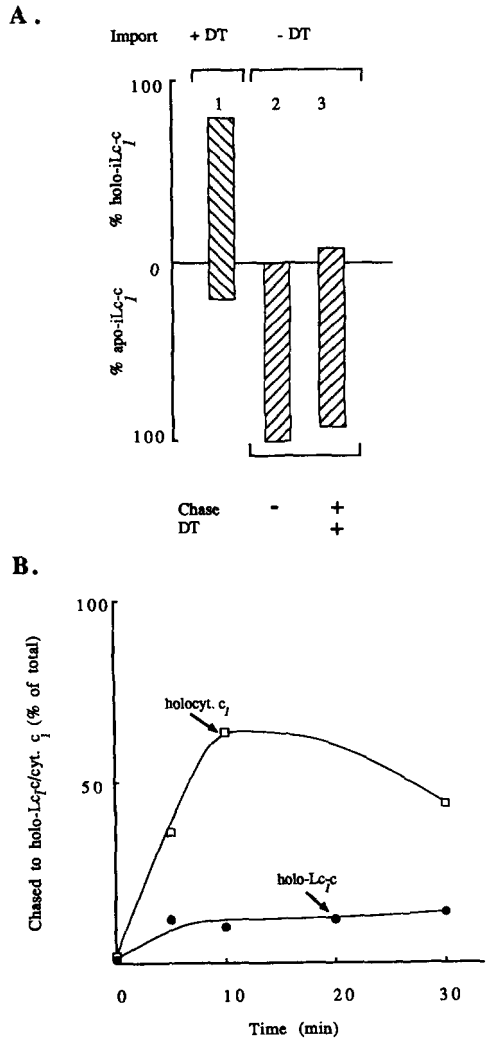

FIG. 7. **Following import, apo-iLc_1-*c* cannot be chased to holo-iLc_1-*c*.** *A*, pLc_1-*c* was synthesized in the presence of [^{35}S] cysteine and was imported into isolated mitochondria either in the presence (*track 1*) or absence of sodium dithionite (1 mg/ml) (*tracks 2* and *3*). Following proteinase K treatment, mitochondria were reisolated by centrifugation. Mitochondria from samples 2 and 3 were resuspended in SME buffer, and aliquots (equivalent to 50 μg 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 Lc_1-*c* was immunoprecipitated. Following reverse-phase HPLC, the amounts of protease-resistant apo-iLc_1-*c* and holo-iLc_1-*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 iLc_1-*c* present as either apo-iLc_1-*c* or holo-iLc_1-*c*. The intermediate size Lc_1-*c* was the only species to accumulate under both of these import conditions (results not shown). *B*, The [^{35}S] cysteine-labeled cytochrome c_1 and pLc_1-*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 $MgCl_2$ at 25 °C as described under "Materials and Methods." After treatment with proteinase K, mitochondria were reisolated 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_1 and Lc_1-*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_1 and Lc_1-*c* were immunoprecipitated, and the amounts of holocytochrome c_1 and holo-iLc_1-*c* formed were determined as described under "Materials and Methods."

cient in cytochrome *c* heme lyase activity (Nargang *et al.*, 1988). Thus, any holo formation occurring in these mitochondria must be a result of cytochrome c_1 heme lyase activity.

Holocytochrome *c* formation occurred in the wild-type mitochondria, independent of a mitochondrial membrane potential; however, no significant cytochrome *c* heme lyase activity was detectable in the *cyt2-1* mitochondria (Fig. 8, *panel A*). The results are thus in agreement with previous findings (Zimmermann *et al.*, 1979; Nargang *et al.*, 1988). Formation of holocytochrome c_1, on the other hand, was dependent on the presence of a $\Delta\Psi$ (as demonstrated previously) and occurred also in the *cyt2-1* mitochondria, thus demonstrating the presence of cytochrome c_1 heme lyase activity in these mitochondria (Fig. 8, *panel B*). Holo-Sc_1-*c* formation was observed in the wild-type mitochondria and, as demonstrated previously, was favored by the absence of a $\Delta\Psi$. No significant holo-Sc_1-*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 $\Delta\Psi$ in wild-type mitochondria had relatively little effect on the holo-Lc_1-*c* formation. More importantly, however, no holo-Lc_1-*c* for-

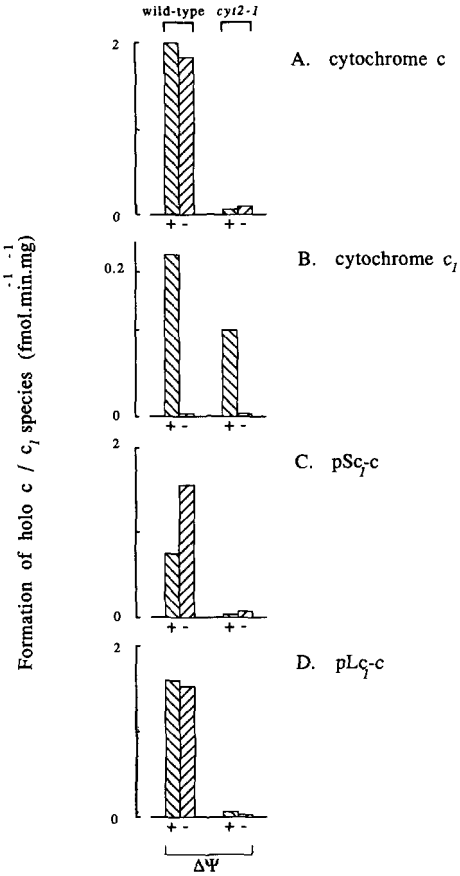

FIG. 8. **pLc_1-*c* and pSc_1-*c* are not substrates for cytochrome c_1 heme lyase.** Apocytochrome *c*, precytochrome c_1, pSc_1-*c*, and pLc_1-*c* were synthesized in reticulocyte lysates in the presence of [^{35}S] cysteine. Mitochondria were isolated freshly from both wild-type and *cty2-1 N. crassa* 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 $MgCl_2$, 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 $-\Delta\Psi$). 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 c_1 (*B*), holo-Sc_1-*c* (*C*), and holo-Lc_1-*c* (*D*) were determined following immunoprecipitated digestion of immunocomplexes with trypsin and subjection 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 pSc_1-*c* and pLc_1-*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 c_1 route (+$\Delta\Psi$), holo-Lc_1-*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 pLc_1-*c* and its processing to iLc_1-*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 pLc_1-*c*, was constructed. pLc_1-*c* contains 3-fold targeting information, in addition to a cytochrome *c* domain, pLc_1-*c* also contain the complete bipartite presequence of the cytochrome c_1 precursor. It has been postulated previously that the cytochrome c_1 presequence contains the necessary information to target this protein not only to the mitochondria but also to its correct submitochondrial location. The proposed import pathways of the pLc_1-*c* are depicted in Fig. 9. The apocytochrome *c* targeting information in pLc_1-*c* was also found to be functional, and consequently pLc_1-*c* could be imported along a cytochrome *c* pathway. This resulted in the accumulation of holo-pLc_1-*c* in the intermembrane space of mitochondria. A functional dissection of the $\Delta\Psi$-dependent import pathway of the pLc_1-*c* fusion protein revealed that the cytochrome c_1 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 c_1 pathway, pLc_1-*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.

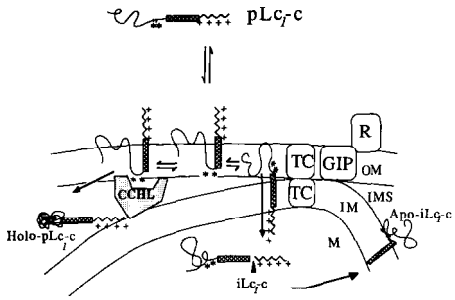

FIG. 9. **Hypothetical import and assembly pathways of pLc_1-*c*.** *A*, import along the cytochrome *c* pathway. The precursor of Lc_1-*c* (pLc_1-*c*) initially inserts into the outer mitochondrial membrane (*OM*) because of the membrane insertion active nature of its apocytochrome *c* domain and forms a salt-resistant complex that includes cytochrome *c* heme lyase (*CCHL*). Upon addition of a reductant, pLc_1-*c* could be imported along a cytochrome *c* pathway where it accumulated in the intermembrane space (*IMS*) as a heme-containing species, holo-pLc_1-*c*. The 2 cysteines to which the heme group becomes attached are indicated by *asterisks*. *B*, import along a cytochrome *c* pathway. On the other hand, pLc_1-*c* could be imported along a cytochrome c_1 route, initially into the matrix (*M*) in a membrane potential-dependent manner. Import of pLc_1-*c* into the matrix via translocation contact sites (*TC*) can occur but is not obligatory. Processing to an intermediate size Lc_1-*c* occurs, catalyzed by the matrix-processing peptidase. This process is followed by retranslocation across the inner membrane (*IM*) whereby iLc_1-*c* becomes exposed to the intermembrane space while being anchored to the inner membrane, presumably via the remaining uncleaved hydrophobic part of its presequence. Retranslocated iLc_1-*c* cannot interact functionally with cytochrome c_1 heme lyase.

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 reexport signal rather than 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 pLc_1-*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 c_1 presequence appears to be responsible for the reexport of the intermediate size cytochrome c_1 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 c_1 presequence and the leader (export) sequences of the bacterial cytochromes c_1 and c_2. 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 pLc_1-*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 became retranslocated across the inner membrane where it was accumulated as an apointermediate size Lc_1-*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. pLc_1-*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_1 presequence and the apocytochrome *c* domain) with the second domain of the cytochrome c_1 presequence, the presence of the cytochrome *c* domain as part of the pLc_1-*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 vincinity of the $\Delta\Psi$-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 pLc_1-*c*, as demonstrated previously for cytochrome c_1, could be accumulated in mitochondria as a translocation contact site intermediate (Schleyer and Neupert, 1985). During import into the matrix of intact mitochondria, pLc_1-*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_1-*c* to insert into the outer membrane spontaneously. Upon doing so, pLc_1-*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[3] (Hwang *et al.*, 1989).

Fourth, although cytochrome *c* cannot be sorted functionally along a conservative sorting pathway, following reexport from the matrix, apo-iLc_1-*c* can be accumulated in the same submitochondrial location as apo-iC_1 (*i.e.* retranslocated into the intermembrane space but remaining attached to the inner membrane). From this position, however, unlike cytochrome c_1, apo-pLc_1-*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_1-*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 c_1 import routes are active), however, holo-iLc_1-*c* can be accumulated. It is proposed here that holo-Lc_1-*c* formation precedes the processing of pLc_1-*c* to iLc_1-*c* by the matrix processing peptidase. Following holo-pLc_1-*c* formation, translocation of pLc_1-*c* across the outer membrane is triggered, thus drawing the protein across, and in doing so the matrix targeting domain gains access to the $\Delta\Psi$-dependent step. Consequently, translocation (at least of the presequence) across the inner membrane occurs, cleavage by matrix processing peptidase then ensues. It is unlikely that the protein finally accumulates in the matrix, as folding of the holocytochrome *c* moiety following heme addition would probably serve to arrest the fusion protein in the membrane system.

Finally, despite the apparent similarity of structural and functional location of the two mitochondrial heme lyases and the structural similarity of the heme binding regions in their respective apoproteins, cytochrome *c* heme lyase and cytochrome c_1 heme lyase have nonoverlapping substrate specificities. It has been shown here that pLc_1-*c* is not a substrate for cytochrome c_1 heme lyase, thus apocytochrome *c* delivered to the intermembrane space via the conservative sorting cytochrome c_1 pathway cannot be converted to holocytochrome *c* by cytochrome c_1 heme lyase. It is feasible, however, that once retranslocated across the inner membrane the conformation of the apocytochrome *c* domain of apo-iLc_1-*c* is not favorable in order to achieve a functional interaction with cytochrome c_1 heme lyase. These results suggest, therefore, that in order to achieve holo formation, apocytochrome *c* must be presented to the intermembrane space from the outside of mitochondria in a particular fashion. If presented to the intermembrane space through the matrix, it cannot interact functionally with the cytochrome *c* heme lyase or cytochrome c_1 heme lyase. We conclude that the two enzymes have different and nonoverlapping substrate specifications. One enzyme (cytochrome c_1 heme lyase) functions for cytochrome c_1 delivered to it through the inner membrane, and the other (cytochrome *c* heme lyase) acts on apocytochrome *c* only when delivered to it across the outer mitochondrial membrane.

[3] J. Rassow and W. Neupert, unpublished results.

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