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Biogenesis of Cytochrome c_1

ROLE OF CYTOCHROME c_1 HEME LYASE AND OF THE TWO PROTEOLYTIC PROCESSING STEPS DURING IMPORT INTO MITOCHONDRIA*

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The biogenesis of cytochrome c_1 involves a number of steps including: synthesis as a precursor with a bipartite signal sequence, transfer across the outer and inner mitochondrial membranes, removal of the first part of the presequence in the matrix, reexport to the outer surface of the inner membrane, covalent addition of heme, and removal of the remainder of the presequence. In this report we have focused on the steps of heme addition, catalyzed by cytochrome c_1 heme lyase, and of proteolytic processing during cytochrome c_1 import into mitochondria. Following translocation from the matrix side to the intermembrane-space side of the inner membrane, apocytochrome c_1 forms a complex with cytochrome c_1 heme lyase, and then holocytochrome c_1 formation occurs. Holocytochrome c_1 formation can also be observed in detergent-solubilized preparations of mitochondria, but only after apocytochrome c_1 has first interacted with cytochrome c_1 heme lyase to produce this complex. Heme linkage takes place on the intermembrane-space side of the inner mitochondrial membrane and is dependent on NADH plus a cytosolic cofactor that can be replaced by flavin nucleotides. NADH and FMN appear to be necessary for reduction of heme prior to its linkage to apocytochrome c_1. The second proteolytic processing of cytochrome c_1 does not take place unless the covalent linkage of heme to apocytochrome c_1 precedes it. On the other hand, the cytochrome c_1 heme lyase reaction itself does not require that processing of the cytochrome c_1 precursor to intermediate size cytochrome c_1 takes place first. In conclusion, cytochrome c_1 heme lyase catalyzes an essential step in the import pathway of cytochrome c_1, but it is not involved in the transmembrane movement of the precursor polypeptide. This is in contrast to the case for cytochrome c in which heme addition is coupled to its transport directly across the outer membrane into the intermembrane space.

Most mitochondrial proteins are encoded by genes in the nucleus and are synthesized on free ribosomes in the cytosol as precursors containing amino-terminal extensions. They are then post-translationally imported into mitochondria along pathways that can be subdivided into a consecutive series of distinct steps and which occur by the following generalized sequence of events (for review, see Nicholson and Neupert, 1988; Pfanner *et al.*, 1988a; Hartl *et al.*, 1989): ATP-dependent unfolding of the precursor polypeptide (Pfanner and Neupert, 1986; Chen and Douglas, 1987; Eilers *et al.*, 1987); binding to specific receptors on the mitochondrial surface (Zwizinski *et al.*, 1983, 1984; Hennig *et al.*, 1983; Riezman *et al.*, 1983; Pfaller and Neupert, 1987); insertion into the outer membrane, assisted by an interaction with a putative "general insertion protein" (Pfanner *et al.*, 1988b; Pfaller *et al.*, 1988); membrane potential-dependent insertion into or through the inner membrane via translocation contact sites where the inner and outer membranes come close enough together to be spanned simultaneously by the imported protein (Schleyer *et al.*, 1982; Pfanner and Neupert, 1985; Schleyer and Neupert, 1985; Schwaiger *et al.*, 1987); removal of the amino-terminal presequence by the chelator-sensitive matrix processing peptidase in a step that is aided by a "processing enhancing protein" (Böhni *et al.*, 1980; Hawlitschek *et al.*, 1988); intramitochondrial sorting and final assembly into functional complexes.

Cytochrome c_1 is an electron-carrying subunit of the cytochrome bc_1 complex (ubiquinol:cytochrome c reductase, EC 1.10.2.2). The import of cytochrome c_1 into mitochondria is particularly interesting since its pathway involves a number of steps in addition to those which are common for most other imported mitochondrial proteins. The cytochrome c_1 precursor (p-C_1)[1] is synthesized with an exceptionally long amino-terminal prepiece that is proteolytically removed in two distinct steps during import (Gasser *et al.*, 1982; Teintze *et al.*, 1982; Schleyer and Neupert, 1985; Ohashi *et al.*, 1982; Sadler *et al.*, 1984; Römisch *et al.*, 1987). The precursor is first transported across both mitochondrial membranes where the first part of the presequence is removed by matrix processing peptidase, thereby generating intermediate size cytochrome c_1 (i-C_1). The protein is then retranslocated back across the inner membrane toward the intermembrane space, after which it is processed by a second processing peptidase to generate mature size cytochrome c_1 (m-C_1) (Hartl *et al.*, 1987) in a step that is dependent on NADH (Teintze *et al.*, 1982; Schleyer and Neupert, 1985; Nicholson *et al.*, 1988a). This "conservative sorting" pathway to the intermembrane space via the matrix has been demonstrated for a number of proteins

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[1] The abbreviations used are: p-C_1, i-C_1, and m-C_1 are precursor, intermediate, and mature size cytochrome c_1, respectively; PMSF, phenylmethylsulfonyl fluoride; CC_1HL, cytochrome c_1 heme lyase; apocytochrome c_1 and holocytochrome c_1, the heme-free and heme-containing forms of cytochrome c_1, respectively; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; Mops, 4-morpholinepropanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

including cytochrome c_1 and the Rieske Fe/S protein of the cytochrome bc_1 complex in *Neurospora crassa* mitochondria and cytochrome b_2 in yeast mitochondria (Hartl *et al.*, 1986, 1987). During or following transport of cytochrome c_1 to the intermembrane-space side of the inner membrane, it becomes anchored to the inner membrane by a hydrophobic stretch of amino acids near the carboxyl terminus (Wakabayashi *et al.*, 1980; Li *et al.*, 1981) and is then assembled with other subunits of the cytochrome bc_1 complex (Crivellone *et al.*, 1988).

During import into mitochondria, heme is covalently attached to the cytochrome c_1 polypeptide by the formation of thioether linkages between the heme vinyl groups at positions 3 and 8 of the porphyrin ring and the thiols of cysteines 110 and 113 (amino acid numbering taken from the primary sequence of the *N. crassa* cytochrome c_1 precursor; Römisch *et al.*, 1987). In this step, the heme-free form of cytochrome c_1 (apocytochrome c_1) is enzymatically converted to the heme-containing species (holocytochrome c_1). It is this heme-attaching reaction during the biogenesis of cytochrome c_1 that we have examined in this report. Up to this point it has not been possible to measure the activity of the enzyme that catalyzes holocytochrome c_1 formation, namely cytochrome c_1 heme lyase (CC_1HL). Here we describe a sensitive HPLC-based method for measuring holocytochrome c_1 formation *in vitro*. Furthermore, we have characterized the requirements for this reaction and describe its relationship to other processing events that occur during cytochrome c_1 import into mitochondria. This complex pathway is compared to the relatively simple import pathway of cytochrome *c*. Remarkably different import pathways have evolved for cytochromes c_1 and *c*, which is rather surprising in light of the many similarities in their functions and in submitochondrial location.

MATERIALS AND METHODS

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). Where indicated, cells were radioactively labeled by growth under the same conditions except that sulfate in the media was reduced to 0.08 mM, and 3 mCi of $^{35}SO_4$ (800–1300 Ci/mmol, Amersham Corp.) were added per liter of culture. The cells were harvested by filtration, and mitochondria were isolated by differential centrifugation (Pfanner and Neupert, 1985) in SEM buffer (250 mM sucrose, 2 mM EDTA, 10 mM Mops/KOH, pH 7.2). A cytosol fraction was prepared from the post-mitochondrial supernatant as described before (Nicholson *et al.*, 1987).

Import of Cytochrome c_1 into Mitochondria

Synthesis of the Cytochrome c_1 Precursor—A full-length cDNA coding for the cytochrome c_1 precursor was isolated from a *N. crassa* library and cloned into pGEM3 as described before (Römisch *et al.*, 1987; Hartl *et al.*, 1987). Isolated plasmids were transcribed with SP6 RNA polymerase (Boehringer Mannheim), and the capped transcripts were used to direct protein synthesis. The precursor of cytochrome c_1 was then synthesized in nuclease-treated rabbit reticulocyte lysates (Pelham and Jackson, 1976) in the presence of L-[^{35}S]cysteine (1100–1400 Ci/mmol, Amersham Corp.) as previously described (Nicholson *et al.*, 1987). Aliquots of the post-ribosomal supernatant were stored at −80 °C under a nitrogen atmosphere.

In order to synthesize the mature size cytochrome c_1 without the presequence, translation was started at a downstream ATG codon (corresponding to methionine 71, the first amino acid residue of mature size cytochrome c_1) in the following way (Maniatis *et al.*, 1982). An insert was excised from the plasmid described above by digestion with *Eco*RI plus *Pst*I. The insert was purified and further digested with *Pvu*I. A 980-bp *Pvu*I-*Pst*I fragment, which contained the coding region for the entire mature cytochrome c_1 but only that part of the presequence proximal to the mature region, was isolated, treated with S1 nuclease and Klenow polymerase, and ligated into the *Sma*I site of pGEM3. Clones oriented in the T7 RNA polymerase direction were selected. Isolated plasmids were transcribed with T7 RNA polymerase, and capped transcripts were used to direct protein synthesis as described above.

Import into Mitochondria—Mitochondria (50 μg of protein) and [^{35}S]cysteine-labeled reticulocyte lysate (10 μl, containing the cytochrome c_1 precursor) were incubated together in a buffer (100 μl final volume) composed of 3% (w/v) bovine serum albumin, 70 mM KCl, 220 mM sucrose, 10 mM Mops/KOH, pH 7.2, plus other additions as indicated. (Note: When more than one sample required the same treatment, pools were prepared that were appropriate multiples of the individual import mixture but were otherwise treated the same.) After 30 min (or as indicated) at 25 °C, the samples were cooled to 0 °C, and proteinase K (Boehringer Mannheim) was added to a final concentration of 20 μg/ml in order to digest nonimported cytochrome c_1. After 30 min at 0 °C, the protease treatment was halted by diluting the samples with 1 ml of SEM buffer containing 2 mM phenylmethylsulfonyl fluoride (PMSF). The mitochondria were then reisolated from the mixtures by centrifugation for 12 min at 17,400 × *g* (Beckman JA-20 rotor).

Post-import Analysis—The mitochondrial pellets were treated in one of two ways depending on whether the processing state of the imported cytochrome c_1 was to be determined or whether the amount converted to holocytochrome c_1 was to be quantified. 1) For determining the amount and relative size of imported (protease-resistant) cytochrome c_1, the mitochondrial pellets were lysed with SDS-containing sample buffer and resolved on SDS-polyacrylamide gels (Laemmli, 1970). Radioactive bands in the dried gel were visualized by fluorography (Amplify, Amersham Corp.) and quantified by laser densitometry of the resulting firm. Where comparisons were made between different size species of cytochrome c_1, a correction was made to take the number of radioactive cysteines contained in them into account (4 cysteines are contained in the precursor, whereas the intermediate and mature sizes contain 3 each). 2) For determining the amounts of apo- and holocytochrome c_1, the mitochondrial pellets were lysed in 1 ml of 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris/HCl, pH 7.2. Cytochrome c_1 was then immunoprecipitated and prepared for analysis by reverse-phase HPLC as described below.

Measurement of Cytochrome c_1 Heme Lyase Activity

Principle of the Assay—The HPLC-based method for determining cytochrome *c* heme lyase activity (Nicholson *et al.*, 1987) was adapted for measuring cytochrome c_1 heme lyase activity. The assay is based on the differential retention of a tryptic peptide of cytochrome c_1, consisting of amino acids 108–118 and containing the heme attachment sites (cysteines 110 and 113), on a reverse-phase HPLC column. When covalently coupled to this peptide, the hydrophobic heme group markedly increases the retention time during reverse-phase HPLC, thereby cleanly separating it from the heme-free (apo) form of the peptide. To radiolabel the peptide, the cytochrome c_1 precursor was synthesized *in vitro* in the presence of [^{35}S]cysteine. Following the incubation of [^{35}S]cysteine-labeled precytochrome c_1 with mitochondria, the cytochrome c_1 products were immunopurified and digested with trypsin to generate peptides. The peptide mixture was resolved by reverse-phase HPLC, and the heme-containing (holo) form of the peptide was collected and its radioactivity measured.

Analysis of Holocytochrome c_1 Formation—Apocytochrome c_1 was synthesized in rabbit reticulocyte lysate in the presence of [^{35}S] cysteine, and the radiolabeled precursor protein was then imported into mitochondria at 25 °C. Unless otherwise indicated, the import mixtures were treated with proteinase K, and the mitochondria were reisolated by centrifugation (see above). The mitochondrial pellets were then prepared for immunoprecipitation by lysing them with 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris/HCl (pH 7.2). Cytochrome c_1 was immunoprecipitated as described previously for cytochrome *c* (Nicholson *et al.*, 1987) except that a specific antiserum directed against cytochrome c_1 was used. The immunocomplexes were eluted from the protein A-Sepharose (Pharmacia) with urea, and tryptic peptides were generated as described before (Nicholson *et al.*, 1987) except that (i) porcine pancreas trypsin was used (40 units/mg, Merck), (ii) the incubation was shortened to 60 min, and (iii) the reaction was halted by adding 25 μg of soybean trypsin inhibitor (Type I-S, Sigma). The resulting peptide mixtures were bound to a μBondapak-phenyl HPLC column (3.9 mm × 30 cm, 10 μm particle size, Waters Associates) that had been equilibrated in 0.1% (v/v) trifluoroacetic acid in water (at a flow rate of 0.8 ml/min), and the peptides were eluted with a linear gradient from 0 to 50% of solvent

B (0.1% trifluoroacetic acid, 9.9% water, 90% acetonitrile (Merck), v/v/v) as described in Fig. 1*C*. Following this procedure, the cysteine-containing peptide comprised of amino acids 108–118 eluted at 38 min (about 15% solvent B), whereas the heme-containing (holo) form of the peptide eluted at 63 min (about 36% solvent B). The latter peptide was collected, and its radioactivity was determined as a measure of holocytochrome c_1 formation. When required, the apopeptide eluting at 38 min was also collected and its radioactivity determined. Using this procedure, formation of holocytochrome c_1 was dependent on the presence of mitochondria and was linear up to 100 μg of mitochondrial protein (100 μl final volume). The reaction was also linear for 15 min (see below) at 25 °C (the optimal temperature). Activity was dependent on NADH (optimal at 5 mM) or sodium dithionite (1 mg/ml) but also occurred in the presence of NADPH (1 mM) or reduced glutathione (40 mM), although with lower efficiency in the latter two cases. The enzyme was active over a broad pH range with an optimum at pH 7.2. Up to 25% of the cytochrome c_1 precursor available in the import mixtures could be taken up by mitochondria and converted to holocytochrome c_1. The variation between replicate samples was less than 5% (data not shown).

Miscellaneous

Published procedures were used for the following: purification of the cytochrome bc_1 complex (Complex III) from isolated *N. crassa* mitochondria (Weiss and Juchs, 1978; Weiss and Kolb, 1979), except that endogenous cytochrome *c* was released from the mitochondria by treatment with digitonin (0.2% (w/v) final concentration, see Nicholson *et al.*, 1987) instead of by sonication; production of specific antibodies in rabbits (Hennig and Neupert, 1983) using the cytochrome c_1 band excised from SDS-polyacrylamide gels as antigen; subfractionation of mitochondria with digitonin following import (Hartl *et al.*, 1986); measurement of adenylate kinase (Schmidt *et al.*, 1984) and fumarase (Kanarek and Hill, 1964) activities; SDS-polyacrylamide gel electrophoresis (Laemmli, 1970); fluorography (Nicholson *et al.*, 1987); radiosequencing analysis by automated solid-phase Edman degradation (Wachter *et al.*, 1973); determination of radioactivity (Nicholson *et al.*, 1987); protein determination (Bradford, 1976); preparation of a *N. crassa* cytosolic protease-inhibitor fraction (Schmidt *et al.*, 1984); import of F_1-ATPase subunit β into mitochondria (Schwaiger *et al.*, 1987); preparation of hemin and deuterohemin (Nicholson and Neupert, 1989); processing of p-cytochrome c_1 (synthesized in reticulocyte lysate) to i-cytochrome c_1 by a purified preparation of the matrix processing peptidase (Hawlitschek *et al.*, 1988). The first proteolytic cleavage site of the cytochrome c_1 presequence was determined by radiosequence analysis (Wachter *et al.*, 1973; Hartl *et al.*, 1986) of a [^{35}S]cysteine-labeled fusion protein (comprised of the first 34 amino acids of the cytochrome c_1 presequence joined to the amino terminus of cytochrome *c*) after it had been processed by matrix processing peptidase in intact mitochondria or by a purified preparation of matrix processing peptidase.[2]

RESULTS

Measurement of Cytochrome c_1 Heme Lyase-Catalyzed Formation of Holocytochrome c_1—We have previously described a method for determining the conversion of apocytochrome *c* to holocytochrome *c*; a reaction catalyzed by the enzyme cytochrome *c* heme lyase (Nicholson *et al.*, 1987). We have adapted the principles of this assay in order to measure the conversion of apocytochrome c_1 to holocytochrome c_1 (Fig. 1). Peptides of cytochrome c_1 could be generated by digestion with trypsin. One of these peptides (amino acids 108–118) contains the 2 cysteine residues to which the heme group becomes covalently attached via thioether linkages (Fig. 1*A*). Since the heme group is hydrophobic, it markedly changes the properties of the peptide when it is present. The two forms of the peptide (apo and holo) could therefore be resolved by reverse-phase HPLC.

As a first step, it was necessary to determine when the respective apo and holo tryptic peptides were eluted from the HPLC column. To do this for the heme-free (apo) form of the peptide, apocytochrome c_1 was synthesized in reticulocyte lysate in the presence of [^{35}S]cysteine and then immunoprecipitated. The immunocomplexes were dissociated with urea, digested with trypsin, and the resulting peptide mixture was resolved by reverse-phase HPLC. A single major radioactive peak eluted from the column at 38 min following sample

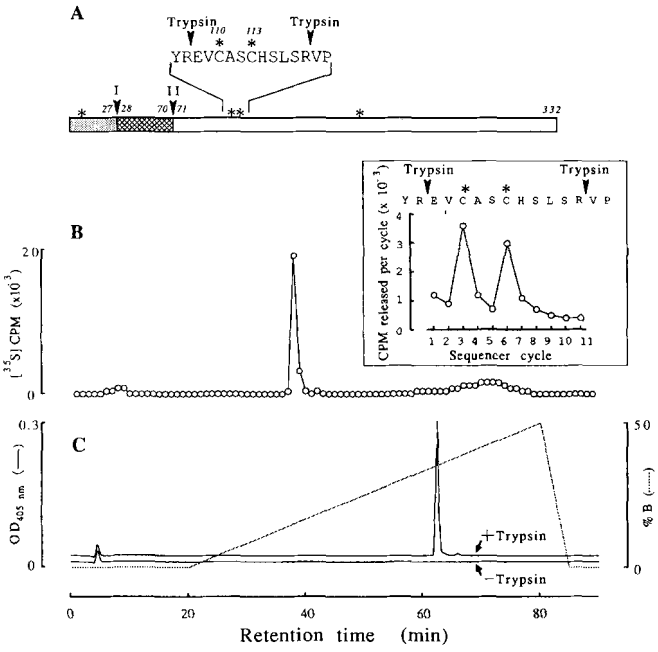


FIG. 1. **Resolution and identification of tryptic peptides of cytochrome c_1 from *N. crassa*.** *A*, schematic representation of the cytochrome c_1 precursor polypeptide. The cytochrome c_1 precursor is depicted in three segments: the first being the part of the prepiece (amino acids 1–27) removed by the chelator-sensitive matrix processing peptidase (cleavage site I); the second being the remainder of the prepiece (amino acids 28–70) which is removed by a second processing peptidase (cleavage site II); and the third being the mature part of cytochrome c_1 (amino acids 71–332). Cysteine residues at positions 6, 110, 113, and 197 are indicated by *asterisks*. The heme-binding region is enlarged and the amino acids flanking cysteines 110 and 113 are shown using the single letter amino acid code. The peptide bonds cleaved by trypsin, thereby generating a fragment containing amino acids 108–118, are indicated by *arrowheads*. The sequence was taken from Römisch *et al.*, 1987. The first cleavage site (between amino acids 27 and 28) was determined as described under "Materials and Methods." *B*, HPLC analysis of tryptic peptide fragments of apocytochrome c_1. The precursor of cytochrome c_1 was synthesized in a reticulocyte lysate in the presence of [^{35}S]cysteine and immunoprecipitated. The immunoprecipitate was dissociated with urea, digested with trypsin and the resulting peptides were resolved by reverse-phase HPLC as described under "Materials and Methods." Samples were collected at 1-min intervals and their radioactivity was determined. *Inset*, The major radioactive peak eluting at 38 min was collected and subjected to radiosequence analysis. The amino acid sequence of the peptide predicted to comprise the 38-min peak (amino acids 108–118, which contains the heme attachment sites) is aligned over the corresponding sequencer cycles. *C*, identification of the heme-containing tryptic peptide of holocytochrome c_1 produced *in vivo*. Complex III from *N. crassa* mitochondria was purified (as described under "Materials and Methods") and then denatured by suspending 0.5 mg of protein in 50 μl of 8 M urea. The sample was shaken for 15 min, incubated for 30 min at 56 °C and then shaken again for 15 min. Peptide fragments were generated by digestion with trypsin as described for immunoprecipitated cytochrome c_1 (see "Materials and Methods") and analyzed by reverse-phase HPLC (*+Trypsin*). The chromatograph was monitored at 405 nm to detect the heme-containing peptide arising from the cytochrome c_1 subunit of complex III. A parallel sample was processed in an identical manner except that no trypsin was added (*−Trypsin*). Undigested cytochrome c_1 was not eluted using the gradient shown but eluted at higher percentages of solvent B (approximately 61%) (not shown).

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

injection (Fig. 1*B*). To positively identify the peptide, this peak was collected and subjected to radiosequencing analysis by solid-phase Edman degradation (Fig. 1*B*, *inset*). Radioactivity was released from the solid-phase support following three and six cycles of Edman degradation. This was as predicted (see peptide sequence aligned at top of Fig. 1*B*, *inset*) and verified the identity of the heme-binding peptide. The peptides containing the other 2 cysteines present in the cytochrome c_1 precursor (amino acids 6 and 197) were not eluted as distinct peaks by the gradient shown and thus did not interfere with detection of the peptide containing the heme binding region.

In order to determine where the heme-containing (holo) form of the peptide eluted, the cytochrome bc_1 complex was purified from *N. crassa*, denatured with urea, and then digested with trypsin. The resulting peptide mixture was resolved by reverse-phase HPLC (Fig. 1*C*) and monitored at 405 nm to detect the heme-containing peptide arising from the cytochrome c_1 subunit (+*Trypsin*). A single peak was detected that had a retention time of 63 min. In the absence of trypsin, no peak absorbing at 405 nm was detected (−*Trypsin*). Under these conditions, undigested cytochrome c_1 was not eluted from the column.

The clear-cut separation of the peptides enabled us to establish an assay system for holocytochrome c_1 formation. Cytochrome c_1 precursor (p-C_1), synthesized in reticulocyte lysate (directed by transcripts from a cDNA clone) in the presence of [^{35}S]cysteine, was imported into isolated mitochondria under various conditions. Following import, the samples were treated with proteinase K to digest what was not taken up by mitochondria. Half of each sample was then used for determining the relative size of the imported cytochrome c_1 species (Fig. 2*A*) while the remaining half was used for analysis of ^{35}S radioactivity in the apo and holo forms of the tryptic peptides containing the heme-binding sites (Fig. 2*B*).

In a first import reaction, EDTA and *o*-phenanthroline (inhibitors of matrix processing peptidase, the matrix-localized processing peptidase) were present. Accumulation of protease-resistant p-C_1 was observed (Fig. 2*A*, *lane I*). The p-C_1 was entirely apocytochrome c_1 since radioactivity was only detected in a peak having a retention time of 38 min (Fig. 2*B*, *trace I*). When import was performed in the presence of $MgCl_2$ (a condition that leads to activation of matrix processing peptidase), cytochrome c_1 was imported and processed to its intermediate size form (i-C_1) (Fig. 2*A*, *lane II*). The i-C_1 was predominantly apocytochrome c_1 (Fig. 2*B*, *trace II*). On the other hand, under identical import conditions except in the presence of NADH, approximately 60% of the imported cytochrome c_1 was mature size (m-C_1), the remainder being intermediate size (Fig. 2*A*, *lane III*). Similarly, approximately 60% was holocytochrome c_1 (Fig. 2*B*, *trace III*). A close relationship between the heme attaching step and the second proteolytic processing step appears to exist. This indicates that NADH at the same time promotes heme attachment and processing to mature size holocytochrome c_1.

The Heme-attaching Step Precedes the Second Proteolytic Processing of Cytochrome c_1—At which stage of the import pathway does covalent heme attachment occur with respect to other processing steps? Cytochrome c_1 was found to be imported into mitochondria and accumulated as either the precursor or intermediate size species (p-C_1 and i-C_1, respectively) in the absence of covalently attached heme (see above). Therefore neither the transport of the cytochrome c_1 precursor into mitochondria (the membrane potential-dependent step) nor removal of the first part of the presequence (by matrix processing peptidase) required prior linkage of heme to apocytochrome c_1. On the other hand, processing to mature-size cytochrome c_1 was dependent on NADH, a requisite for cytochrome c_1 heme lyase (CC_1HL) activity (see below), suggesting that heme attachment must precede the second proteolytic processing event during import.

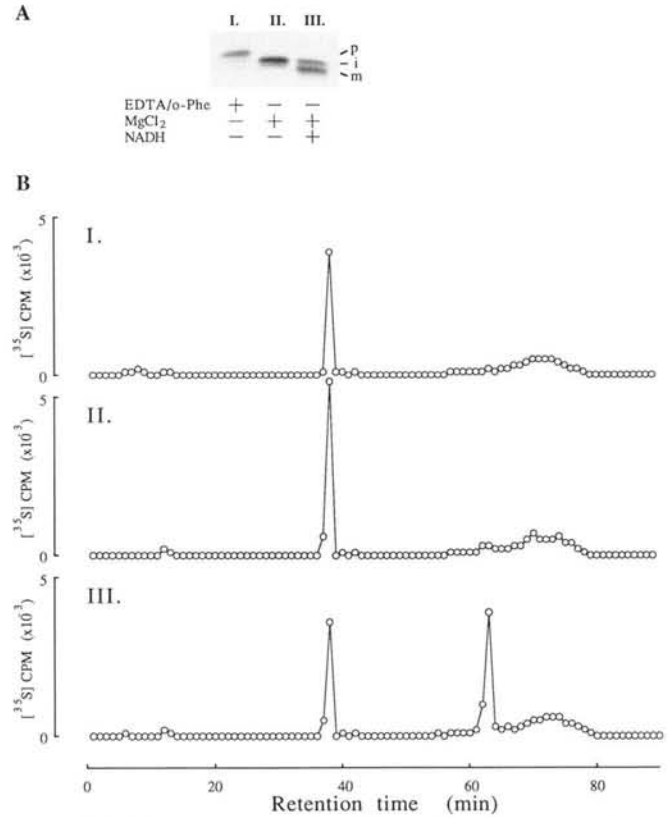


FIG. 2. **HPLC analysis of tryptic peptides of imported cytochrome c_1 species.** The precursor of cytochrome c_1 was synthesized *in vitro* in the presence of [^{35}S]cysteine and then imported into mitochondria in the presence of 5 mM EDTA plus 0.2 mM *o*-phenanthroline (*I*), 2.5 mM $MgCl_2$ (*II* and *III*) or 5 mM NADH (*III*) as described under "Materials and Methods." Following proteinase K treatment, the samples were divided and the mitochondria were reisolated by centrifugation. One of the portions from each sample was analyzed by SDS-gel electrophoresis and fluorography (*panel A*). Cytochrome c_1 was immunoprecipitated from the remaining half and digested with trypsin. The resulting peptide mixtures were resolved by reverse-phase HPLC. Fractions were collected at 1-min intervals, and their radioactivity was determined (*panel B*). *p*, *i*, and *m* are precursor, intermediate, and mature size cytochrome c_1, respectively. *o*-Phe, *o*-phenanthroline.

The kinetics of accumulation of distinct import intermediates was determined under various conditions that selectively trapped cytochrome c_1 at specific stages of its import pathway (Fig. 3). In the absence of NADH, cytochrome c_1 was rapidly imported (Fig. 3*A*). When processing by matrix processing peptidase was inhibited by EDTA plus *o*-phenanthroline (comparable to *lane I* of Fig. 2*A*), p-C_1 was imported to a protease-resistant location reaching half-maximal accumulation in approximately 2.5 min. Under conditions where matrix processing peptidase was fully active (in the presence of $MgCl_2$, comparable to *lane II* of Fig. 2*A*), the cytochrome c_1 precursor was imported and processed to i-C_1, requiring about 6 min for half-maximal accumulation. In both of these cases, heme was not attached to either p-C_1 or i-C_1 (not shown; *cf.* Fig. 2*B*).

In the presence of NADH and $MgCl_2$ (comparable to *lane III* of Fig. 2*A*), intermediate size cytochrome c_1 was rapidly

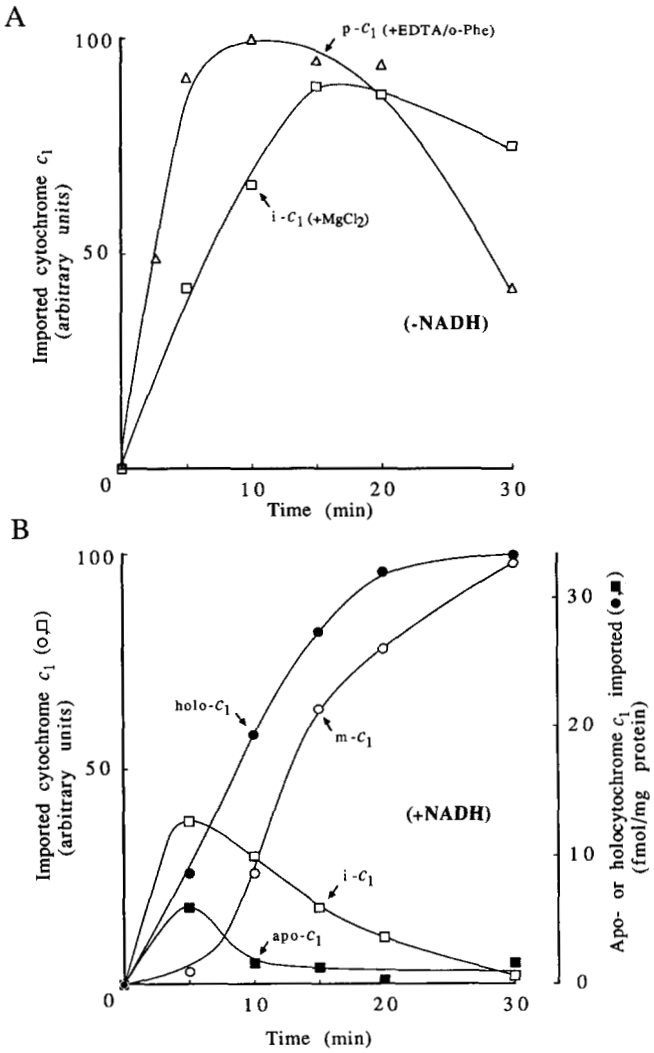


FIG. 3. **Kinetics of cytochrome c_1 import and holocytochrome c_1 formation.** The cytochrome c_1 precursor was synthesized *in vitro* in the presence of [^{35}S]cysteine and then imported into mitochondria for varying periods of time under the conditions described in Fig. 2. *A*, accumulation of protease-resistant precursor size (p-C_1) or intermediate size (i-C_1) cytochrome c_1. Mitochondria (50 μg of protein) were incubated with [^{35}S]cysteine-labeled cytochrome c_1 precursor at 25 °C for the indicated time periods in the presence of either 5 mM EDTA plus 0.2 mM *o*-phenanthroline (△) or in the presence of 2.5 mM $MgCl_2$ (□). Following treatment with proteinase K, the mitochondria were reisolated by centrifugation (see "Materials and Methods"). The resulting mitochondrial pellets were lysed in SDS-containing sample buffer and resolved on SDS-polyacrylamide gels. The cytochrome c_1 bands from a fluorogram of the resulting gel were quantified by laser densitometry. *B*, cytochrome c_1 import and holocytochrome c_1 formation in the presence of NADH. Double size import mixtures were prepared containing mitochondria (100 μg of protein), [^{35}S]cysteine-labeled cytochrome c_1 precursor, 2.5 mM $MgCl_2$, and 5 mM NADH, and were incubated at 25 °C for the indicated time periods. After cooling to 0 °C, the samples were treated with proteinase K, divided in two and the mitochondria were reisolated by centrifugation. The mitochondrial pellets from one of the aliquots were prepared for SDS-polyacrylamide gels and fluorography. The resulting intermediate-size (i-C_1, □) and mature-size (m-C_1, ○) cytochrome c_1 bands were then quantified as above. Cytochrome c_1 was immunoprecipitated from the remaining group of mitochondrial pellets and the immunocomplexes were dissociated with urea. Peptides were generated by digestion with trypsin and resolved by reverse-phase HPLC. The amounts of apocytochrome c_1 (apo-C_1, ■) and holocytochrome c_1 (holo-C_1, ●) were quantified by collecting the corresponding peptides and determining their radioactivity.

formed but levels decreased with incubation times longer than 5 min (Fig. 3*B*). Mature-size cytochrome c_1 appeared only after a lag of 7–8 min. Processing by matrix processing peptidase was rapid since no protease-resistant p-C_1 was observed (not shown). Covalent attachment of heme was also rapid since even after only 5 min of incubation the imported cytochrome c_1, which was predominantly i-C_1, had been largely converted to holocytochrome c_1 (Fig. 3*B*). Formation of holocytochrome c_1 occurred shortly following transport of cytochrome c_1 into mitochondria (half-maximal at 9 min) and preceded the subsequent processing to m-C_1 (half-maximal at 13 min).

We conclude that import and processing of cytochrome c_1 occurs in the following sequence: transport of p-C_1 into mitochondria to a protease-resistant location (half-maximal at 2.5 min); processing by matrix processing peptidase to yield i-C_1 (half-maximal at 6 min); covalent attachment of the heme group to apocytochrome c_1 (half-maximal at 9 min); cleavage by a second processing peptidase to produce m-C_1 (half-maximal at 13 min).

Since processing to m-C_1 dose not take place in the absence of NADH, addition of the heme group may be obligatory for the second proteolytic processing step to occur. To exclude the possibility that NADH was required for both the CC_1HL reaction and the second processing step, formation of holocytochrome c_1 was inhibited by the analogue deuterohemin to examine whether processing to m-C_1 still occurred with NADH present (Fig. 4) (deuterohemin is an analogue of heme which does not contain the vinyl groups at positions 3 and 8 of the porphyrin ring that are necessary for formation of the thioether linkages with apocytochrome c_1). In the presence of low concentrations of deuterohemin, processing of i-C_1 to m-C_1 was inhibited despite the presence of NADH (Fig. 4*A*, *lanes 2–6*). In fact, with increasing concentrations of deuterohemin, the ratio of m-C_1/i-C_1 in mitochondria was nearly identical to the ratio of holocytochrome c_1/apocytochrome c_1 (Fig. 4*B*). Therefore, inhibition of holocytochrome c_1 formation with deuterohemin also inhibited the processing of i-C_1 to m-C_1. Total cytochrome c_1 import was reduced at higher (*i.e.* >25 μM) concentrations of deuterohemin, presumably owing to nonspecific effects such as perturbation of the membrane potential; however, the relative ratios of m-C_1/i-C_1 compared to holo-C_1/apo-C_1 changed in parallel throughout the entire concentration range tested.

We conclude that during cytochrome c_1 import into mitochondria the formation of holocytochrome c_1 follows shortly after (or during) the processing of p-C_1 to i-C_1 by matrix processing peptidase, but precedes the second proteolytic processing step in which i-C_1 is cleaved to m-C_1. Furthermore, we conclude that covalent attachment of the heme group is mandatory before processing to m-C_1 can occur. This latter finding is substantiated by earlier observations in yeast strains that cannot produce holocytochrome c_1 *in vivo*. Heme-deficient mutants of yeast were unable to produce mature size cytochrome c_1, but instead accumulated i-C_1 in their mitochondria (Gasser *et al.*, 1982; Ohashi *et al.*, 1982). Similarly, a mutant of yeast that is deficient in CC_1HL activity shows an identical phenotype.[3] It appears likely that cytochrome c_1 becomes reversibly stalled along its import pathway when heme attachment cannot take place.

Removal of the Entire Bipartite Cytochrome c_1 Presequence Can Occur in a Single Step in a Reaction That Is Dependent on Holocytochrome c_1 Formation—We asked whether imported cytochrome c_1 has to be cleaved by matrix processing peptidase to i-C_1 before it is a competent substrate for CC_1HL.

[3] A. Haid, personal communication.

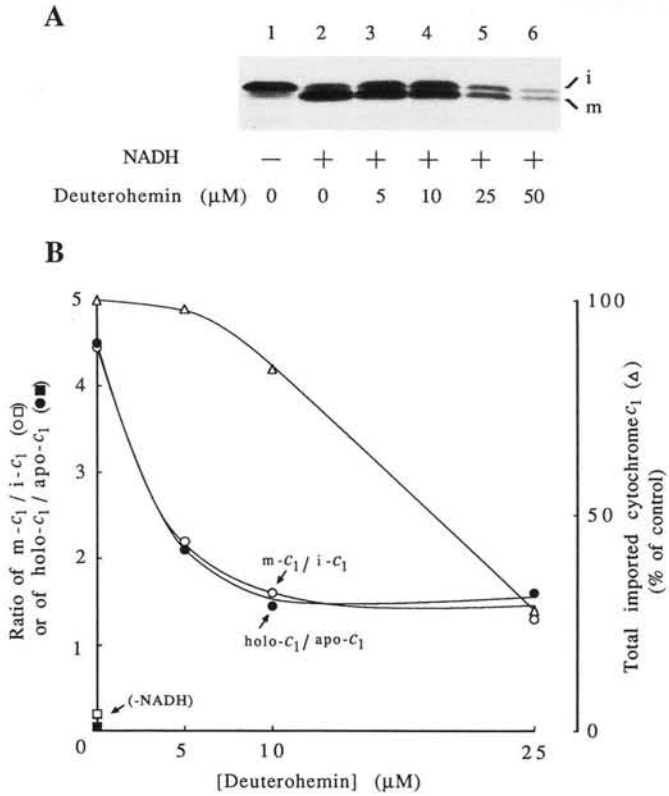


FIG. 4. **Inhibition of both holocytochrome c_1 formation and processing to mature size cytochrome c_1 by deuterohemin.** Double size import mixtures were prepared containing mitochondria (100 μg of protein), [^{35}S]cysteine-labeled cytochrome c_1 precursor, 2.5 mM $MgCl_2$, and varying concentrations of deuterohemin. After 5 min at 0 °C (to allow the deuterohemin to be taken up by mitochondria), NADH (5 mM final concentration) was added (except for *lane 1* of *panel A* and □, ■ of *panel B*). The samples were incubated for 20 min at 25 °C, treated with proteinase K (see "Materials and Methods"), divided, and the mitochondria were reisolated by centrifugation. The mitochondrial pellets from one of the halves were lysed in SDS-containing buffer and resolved on SDS-polyacrylamide gels. The cytochrome c_1 bands from a fluorogram of the resulting gel are shown in *panel A*. The bands were quantified by laser densitometry and the ratio of mature size (m-C_1) divided by intermediate size (i-C_1) cytochrome c_1 is shown in *panel B* (○, □). Total imported cytochrome c_1 was determined by adding the values for both bands together (△). The remaining half of each sample was used to determine the amounts of the imported cytochrome c_1 present as apocytochrome c_1 (apo-C_1) and holocytochrome c_1 (holo-C_1) by reverse-phase HPLC as described in Fig. 3. The ratio of holocytochrome c_1 present divided by that of apocytochrome c_1 is shown (●, ■). Formation of holocytochrome c_1 in the sample corresponding to *lane 2* (*panel A*) was 0.17 fmol·min^{-1}·mg^{-1}.

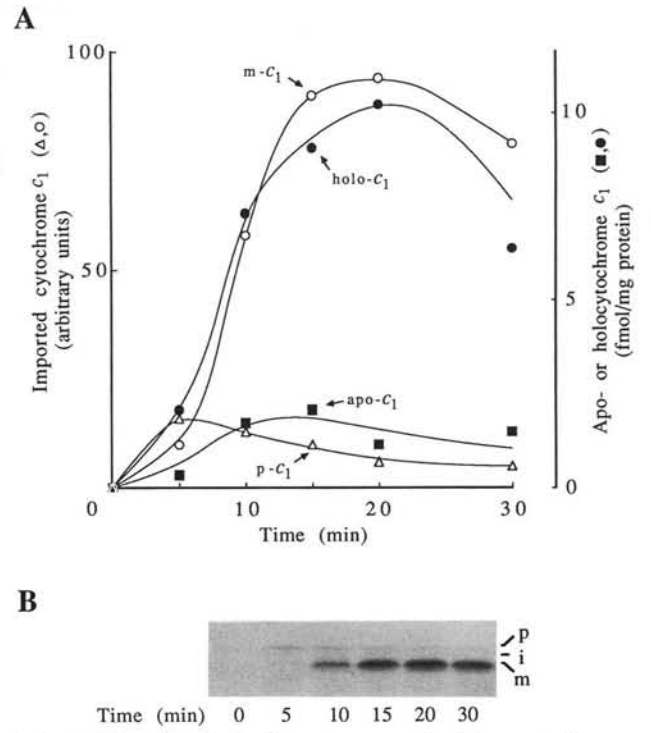


FIG. 5. **Kinetics of single step removal of the cytochrome c_1 prepiece and of holocytochrome c_1 formation.** Mitochondria were preincubated in the presence of 5 mM EDTA plus 0.2 mM *o*-phenanthroline (to inhibit matrix processing peptidase) for 5 min at 0 °C then combined with mixtures containing the same concentrations of EDTA and *o*-phenanthroline plus [^{35}S]cysteine-labeled reticulocyte lysate and 5 mM NADH. The samples (each a double size import mixture) were incubated for varying periods of time at 25 °C (as indicated), cooled to 0 °C then treated with proteinase K (see "Materials and Methods"). After adding PMSF, the samples were divided, and the mitochondria were reisolated by centrifugation. One portion from each sample was prepared for SDS-gel electrophoresis and fluorography. The cytochrome c_1 bands from a fluorogram of the resulting gel are shown in *panel B*. The bands corresponding to precursor size (p-C_1) and mature size (m-C_1) cytochrome c_1 were quantified by laser densitometry (*panel A*, Δ and ○, respectively). The remaining half of each sample was used to determine the amounts of the imported cytochrome c_1 present as apocytochrome c_1 (apo-C_1, ■) or holocytochrome c_1 (holo-C_1, ●) by reverse-phase HPLC (*panel A*) as described in Fig. 3.

To answer this, isolated mitochondria were treated with EDTA plus *o*-phenanthroline to inhibit matrix processing peptidase, and then the cytochrome c_1 precursor was imported into them in the presence of NADH. Substantial holocytochrome c_1 formation did occur (Fig. 5*A*). Remarkably, however, the cytochrome c_1 imported under these conditions was rapidly processed directly from p-C_1 to m-C_1 without any detectable matrix processing peptidase-generated i-C_1 (Fig. 5*B*). The time course of processing to m-C_1 was nearly identical to that of holocytochrome c_1 formation (Fig. 5*A*) indicating a close coupling of the two events. In addition, the kinetics of holocytochrome c_1 formation in the absence of matrix processing peptidase activity was comparable to that in the presence of processing activity (*cf.* Fig. 3*B*). As a control for the possibility that NADH simply reversed the inhibition of matrix processing peptidase by chelators, the import and processing of the precursor of F_1-ATPase subunit β was examined. Processing by matrix processing peptidase to mature F_1-ATPase subunit β, but not the import of precursor F_1-ATPase subunit β, was inhibited by EDTA plus *o*-phenanthroline. Inhibition of processing, however, was not reversed by the presence of NADH (not shown).

We conclude that cytochrome c_1 heme lyase can recognize and covalently attach heme to either p-C_1 or i-C_1. In both cases, the CC_1HL reaction is immediately followed by processing to m-C_1; in the former instance resulting in single step removal of the presequence. Processing by matrix processing peptidase is therefore not obligatory for the correct and efficient import of cytochrome c_1 into mitochondria.

Following Import and Translocation to the Intermembrane Space, Apocytochrome c_1 Forms a Complex with Cytochrome c_1 Heme Lyase—For the other cytochrome in mitochondria that contains covalently bound heme, namely cytochrome *c*, we have previously shown that the apoprotein when bound to mitochondria in an import-competent fashion forms a stable complex with cytochrome *c* heme lyase (Nicholson *et al.*, 1988b). Formation of the complex was highly sensitive to salt, but once produced it was not dissociated with even high

concentrations of salt. Apocytochrome *c* within such a complex could be efficiently chased to holocytochrome *c* in the presence of up to 0.5 M KCl. A similar approach was therefore used to define at which stage of the cytochrome c_1 import pathway the apocytochrome c_1 interacts with CC_1HL.

The cytochrome c_1 precursor was imported into isolated mitochondria under various conditions which allowed the accumulation of distinct transport intermediates. In all cases, NADH was omitted so that holocytochrome c_1 formation did not take place. Varying concentrations of KCl were added subsequently, and the accumulated transport intermediates were then chased in the presence of NADH to allow holocytochrome c_1 formation to occur (Fig. 6). (Prior to the chase incubation, further import was usually blocked by treatment of the mitochondria with proteinase K, except in cases where import of the free precursor or chase from a surface-bound position were being examined. At this time, aliquots were also removed in order to determine the submitochondrial location of the trapped import intermediate by digitonin fractionation.) Two distinct types of import intermediates were observed (Fig. 6*A*): with one type, the chase to holocytochrome c_1 was inhibited by the presence of KCl; with the second type, holocytochrome c_1 formation was not significantly affected by even high concentrations of KCl. Cytochrome c_1 import intermediates belonging to this latter group had apparently interacted with the CC_1HL enzyme in such a way that the subsequent chase to holocytochrome c_1 was not impeded by KCl. This could therefore be used as a criterion for defining at which stage of the cytochrome c_1 import pathway that CC_1HL comes into play. An interpretive model indicating the submitochondrial location of the accumulated import intermediates and their position with respect to CC_1HL is given in Fig. 6*B*; a summary of the methods for accumulating the intermediates, their submitochondrial location and their chaseability to holocytochrome c_1 in the presence of either 0.5 M KCl or after lysis with octyl glucoside (see below) is presented in Table I. For simplicity, import intermediates are arbitrarily numbered I–IV.

Formation of holocytochrome c_1 from the free extramitochondrial precursor (stage I) was impeded by KCl. This is not surprising since any one of a number of import steps, such as receptor binding or membrane potential-dependent entry into mitochondria, may be influenced by salt. Similarly, the chase of surface (receptor)-bound apocytochrome c_1 (stage II) to holocytochrome c_1 was also inhibited by KCl. The chase of cytochrome c_1 precursor which had been trapped on the matrix side of the inner membrane (stage III) by import at reduced temperature (10 °C) showed similar salt sensitivity. In these cases, imported cytochrome c_1 had apparently not yet interacted with CC_1HL.

In the presence of matrix processing peptidase activity, i-C_1 could not be accumulated on the matrix side of the inner membrane by import at low temperature (stage IV), in contrast to p-C_1 accumulated under the same conditions but in the absence of matrix processing peptidase activity (stage III). Removal of the first half of the cytochrome c_1 presequence (by matrix processing peptidase) may therefore enhance the retranslocation step from the matrix to the intermembrane-space side of the inner membrane. Intermediate size cytochrome c_1 imported under these conditions (stage IV) formed a salt-resistant complex with CC_1HL from which it could be chased to holocytochrome c_1 in the presence of KCl. Similarly, when p-C_1 was imported to the matrix side of the inner membrane at 10 °C then retranslocated to the intermembrane-space side by warming to 25 °C (stage III′), the p-C_1 also formed a salt-resistant complex with CC_1HL.

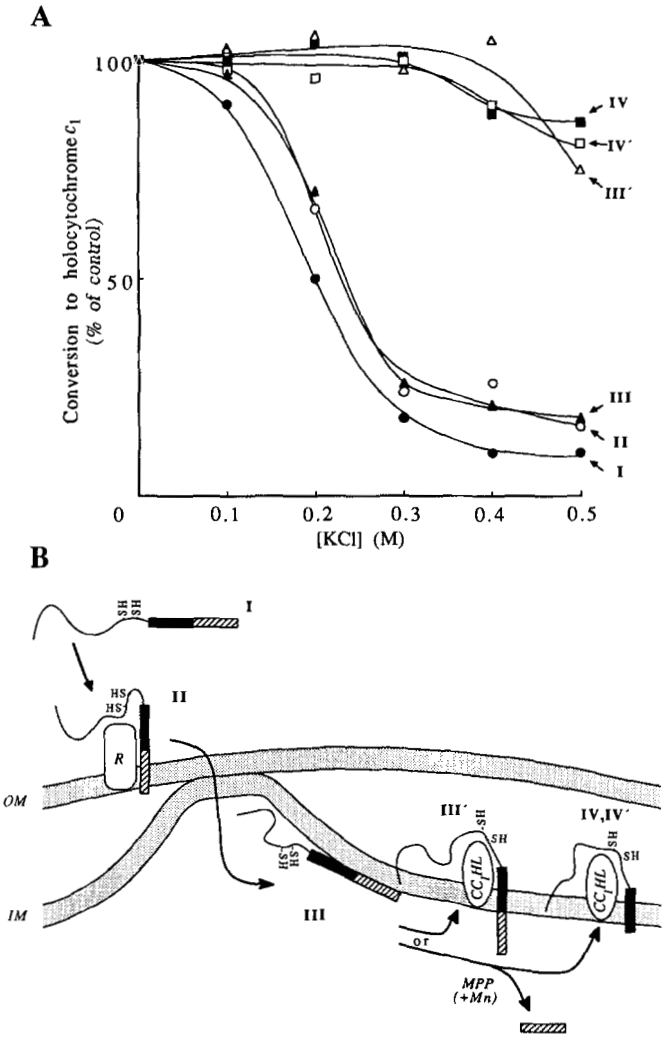


FIG. 6. **Identification of cytochrome c_1 import intermediates competent for conversion to holocytochrome c_1 in the presence of KCl.** Cytochrome c_1 was imported into mitochondria under various conditions and accumulated at distinct stages of its import pathway (arbitrarily named here as stages I–IV). The mitochondria were reisolated (except as noted) and then the accumulated import intermediate was chased to holocytochrome c_1 in the presence of varying concentrations of KCl. *A*, chase of cytochrome c_1 import intermediates to holocytochrome c_1 in the presence of KCl. The sensitivity to KCl of holocytochrome c_1 formation using the free *in vitro*-synthesized precursor (*stage I*, ●) was tested by incubating [^{35}S] cysteine-labeled cytochrome c_1 with mitochondria (50 μg of protein) in the presence of 3 μM hemin, 2.5 mM $MnCl_2$, varying concentrations of KCl (as indicated), and 1 mg of sodium dithionite/ml (added last) for 15 min at 25 °C in a final volume of 100 μl of 0.25 M sucrose, 10 mM Mops/KOH (pH 7.2). Cytochrome c_1 was then immunoprecipitated, and the holocytochrome c_1 formed was determined as described under "Materials and Methods." For the remaining groups (*II–IV*), apocytochrome c_1 was first accumulated as follows. Pools were prepared (*i.e.* multiples of individual import mixtures) containing the equivalent of 50 μg of mitochondrial protein in a final volume of 100 μl of bovine serum albumin-containing buffer (see "Materials and Methods"). In addition, mixture II (○) contained 8 μM antimycin A plus 20 μM oligomycin to block formation of a membrane potential, mixtures III and III′ (▲ and △, respectively) contained 5 mM EDTA plus 0.2 mM *o*-phenanthroline (to inhibit matrix processing peptidase), whereas mixtures IV and IV′ (■ and □, respectively) contained 2.5 mM $MnCl_2$ to support matrix processing peptidase activity. The pools were then incubated for 30 min at 10 °C (incubation at 10 °C impedes retranslocation from the mitochondrial matrix; see Hartl *et al.*, 1986, 1987). Mixtures III′ and IV′ were warmed briefly (5 min) to 25 °C. All of the mixtures were cooled to 0 °C and then treated with proteinase K (except for mixture II which did not receive proteinase K, so as not to degrade the surface-bound cytochrome c_1,

TABLE I

Holocytochrome c_1 formation from accumulated import intermediates

The precursor to cytochrome c_1 was incubated with mitochondria under a variety of defined conditions that lead to accumulation of translocation intermediates at distinct stages of the import pathway as described in Fig. 6. The submitochondrial location of the import intermediates was determined by digitonin fractionation as described under "Materials and Methods." Holocytochrome c_1 formation was then allowed to occur in chase incubations by releasing the block in either the presence or absence of 0.5 M KCl or following lysis of mitochondria with octyl glucoside. The import intermediate was considered chaseable (+) when more than 20% could be converted to holocytochrome c_1 in the presence of 0.5 M KCl or following octyl glucoside lysis, as compared to the untreated control. M, matrix; IMS, intermembrane space; OG, octyl glucoside; *o*-Phe, *o*-phenanthroline.

Stage	Method of accumulating import intermediate		Mitochondrial location	Holocytochrome c_1 formed during chase	Chaseable in presence of 0.5 M KCl	Chaseable after OG lysis
				fmol/mg protein	%	%
I	Free precursor		Extra-mitochondrial	18.5	− (9.5)	− (0)
II	Bound minus membrane potential		Outer surface of outer membrane	6.85	− (16.2)	− (12.4)
III	Imported at 10 °C	EDTA/*o*-Phe	M-side of inner membrane	16.6	− (17.6)	− (9.4)
III	Imported at 10 °C, warmed to 25 °C	EDTA/*o*-Phe	IMS-side of inner membrane	10.4	+ (75.0)	+ (54.2)
IV	Imported at 10 °C	Mn^{2+}	IMS-side of inner membrane	13.6	+ (85.6)	+ (61.6)
IV	Imported at 10 °C, warmed to 25 °C	Mn^{2+}	IMS-side of inner membrane	8.2	+ (82.1)	+ (66.0)

We conclude that apocytochrome c_1 forms a complex with CC_1HL following the translocation of newly imported cytochrome c_1 to the intermembrane-space side of the inner membrane. Furthermore, both p-C_1 and i-C_1 are suitable substrates for this interaction with CC_1HL. We also suggest that CC_1HL is topologically exposed to the intermembrane space since holocytochrome c_1 formation occurs only after the retranslocation of apocytochrome c_1 back across the inner membrane. CC_1HL appears to be membrane-bound since release of the soluble intermembrane space components by exposure to mitochondria to low concentrations of digitonin did not affect the ability of these treated mitochondria to form holocytochrome c_1 (not shown).

Requirements of the Heme-attaching Step of Cytochrome c_1 Biogenesis: Holocytochrome c_1 Formation Is Dependent on NADH and Flavin Nucleotides—In order to examine the CC_1HL reaction independently of other import steps, apo/intermediate size cytochrome c_1 was accumulated at the stage where it forms a complex with CC_1HL (stage IV intermediate in Fig. 6). Requirements for the subsequent formation of holocytochrome c_1 were then tested.

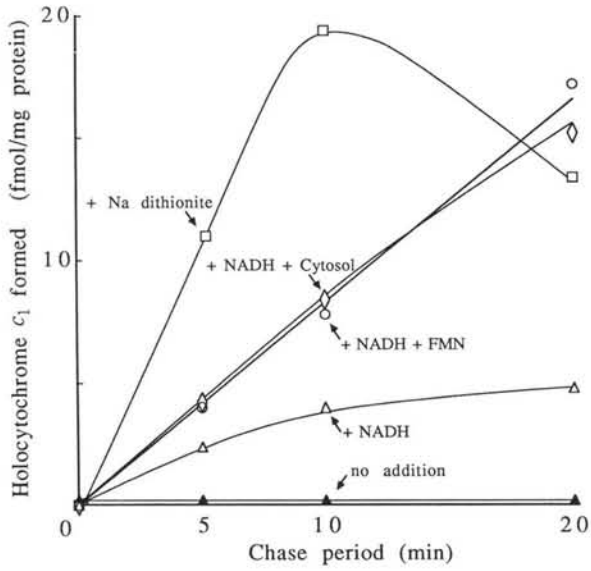
No holocytochrome c_1 formation occurred in the subsequent chase incubations if reducing agents were omitted. In the presence of NADH, however, heme attachment to accumulated apo(i-C_1) did occur (Fig. 7*A*). NADH-dependent holocytochrome c_1 formation was stimulated markedly by the presence of a *N. crassa* cytosol fraction (cytosol fraction alone did not support holocytochrome c_1 formation in the presence or absence of NADH; not shown), and the cytosolic factor could be replaced by FMN (or FAD; not shown). The physical properties of the cytosolic cofactor were identical to those of FMN: both were heat-stable, dialyzable, and sensitive to light (not shown). The presence of dithionite during the chase also substantially stimulated holocytochrome c_1 formation.

We have previously demonstrated that the heme attaching step in the case of cytochrome *c* is also dependent on NADH plus a cofactor present in a cytosol fraction from *N. crassa* or in reticulocyte lysate (Nicholson *et al.*, 1988a). In addition, we have identified this cofactor to be a flavin nucleotide and have shown that NADH, in concert with flavin nucleotides, mediates the reduction of heme; a prerequisite for the covalent attachment of the heme group to apocytochrome *c* (Nicholson and Neupert, 1989). Since the requirement for NADH and FMN is identical for the covalent linkage of heme to apocytochrome c_1, we suggest that heme in the reduced state is also necessary for holocytochrome c_1 formation.

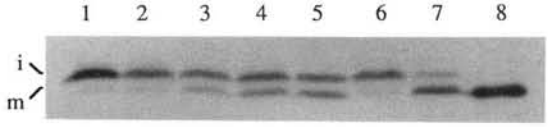
The covalent attachment of heme to accumulated apo(i-C_1) in the presence of reducing agents was accompanied by processing of i-C_1 to m-C_1 (not shown). These observations support the above conclusion that the second proteolytic processing step is dependent on prior formation of holocytochrome c_1. The same requirement was observed for m-C_1 formation from i-C_1 that had been accumulated *in vivo* (Fig. 7*B*). Cells were metabolically labeled *in vivo* by growth at 8 °C in the presence of [^{3}H]leucine. The mitochondria were isolated and examined for processing of accumulated i-C_1 (*lane 1*) to m-C_1 under various conditions *in vitro*. Processing to m-C_1 was dependent on NADH (*lanes 3–5*), reflecting the requirement for holocytochrome c_1 formation and was further enhanced by KCl (*lane 7*). These results again confirm the close rela-

but was otherwise treated the same). Proteinase K activity was inhibited with PMSF, and the mitochondria were reisolated by centrifugation (see "Materials and Methods"). The mitochondrial pellets were resuspended in 0.25 M sucrose, 10 mM Mops/KOH (pH 7.2) containing 1 mg of a *Neurospora* protease-inhibitor fraction/ml. A portion of each mixture was removed for localization of the accumulated import intermediate by digitonin fractionation as described under "Materials and Methods." The remainder was chased to form holocytochrome c_1 in mixtures containing 50 μg of mitochondrial protein (having the accumulated cytochrome c_1 import intermediate), varying concentrations of KCl (as indicated), 3 μM hemin and 1 mg of sodium dithionite/ml (added last) in a final volume of 100 μl of 0.25 M sucrose, 10 mM Mops/KOH (pH 7.2). In addition, samples from mixture II contained 0.2 mM *N,N,N′,N′*-tetramethylphenylenediamine plus 8 mM potassium ascorbate (from a stock adjusted to pH 7.2 with KOH) to reestablish the membrane potential; samples from mixtures II, III, and III′ received 2.5 mM $MnCl_2$ to activate matrix processing peptidase. All samples were incubated for 15 min at 25 °C after which the holocytochrome c_1 that was formed was determined as described under "Materials and Methods." *B*, interpretive model (submitochondrial localization, processing state, and relationship to CC_1HL) of accumulated cytochrome c_1 import intermediates. Import intermediates of cytochrome c_1 (arbitrarily named I–IV) were accumulated (*panel A*), localized by digitonin fractionation, and their state of processing determined by SDS-gel electrophoresis and fluorography (not shown). The cytochrome c_1 prepiece is depicted by a *rectangle*; the *lightly hatched part* being the amino terminus which is removed by matrix processing peptidase and the *densely hatched part* the remainder of the prepiece. The cysteine sulfhydryl groups to which heme becomes attached are indicated. *OM*, outer membrane; *IM*, inner membrane; *R*, receptor; *CC_1HL*, cytochrome c_1 heme lyase; *MPP*, matrix-localized processing peptidase.

A



B



	0°C						
[NADH] (mM)	—	—	0.5	1	2	—	1
[NAD^+] (mM)	—	—	—	—	—	1	—
[KCl] (mM)	—	—	—	—	—	—	80
% m-c_1	0	14	27	39	46	15	74

FIG. 7. **Reduction requirements for cytochrome c_1 import subsequent to the membrane potential-dependent step.** *A*, reduction requirements for holocytochrome c_1 formation from intermediate size cytochrome c_1 accumulated *in vitro*. The [^{35}S]cysteine-labeled cytochrome c_1 precursor was imported into mitochondria to its intermediate size form in a pool equivalent to 50 μg of mitochondrial protein per sample in the presence of 2.5 mM $MgCl_2$ for 20 min at 25 °C as described under "Materials and Methods." After treatment with proteinase K, the mitochondria were reisolated by centrifugation and suspended in SEM buffer (containing 1 mM PMSF) at a protein concentration of 2 mg/ml and portioned into aliquots containing 50 μg of mitochondrial protein each. The intermediate size cytochrome c_1 was the predominant species (*i.e.* >95%) accumulated under these import conditions (not shown; see Fig. 4*A*, *lane 1*, for comparison). The imported intermediate size cytochrome c_1 was then chased for varying periods of time (as indicated) at 25 °C in mixtures (100 μl final volume in SEM buffer) without further addition (▲) or that contained 5 mM NADH (△, ○, ◇), 10 μM FMN (○), 20% (v/v) *N. crassa* cytosol fraction (◇), or 1 mg of sodium dithionite/ml (□). After the incubation period, cytochrome c_1 was immunoprecipitated, and the amount of holocytochrome c_1 that was formed was determined as described under "Materials and Methods." Note: under optimal conditions (after chasing for 10 min in the presence of sodium dithionite; □), 64.2% of the imported cytochrome c_1 was converted to holocytochrome c_1. *B*, intermediate size cytochrome c_1 accumulated *in vivo* is chased to mature size cytochrome c_1 *in vitro* in the presence of NADH. *N. crassa* cells were grown for 15 h at 25 °C as described under "Materials and Methods." A portion of the culture (containing 0.3 g of cells) was removed and cooled to 8 °C. The cells were labeled by adding 0.75 mCi of [^{3}H]leucine and incubating for 10 min at 8 °C. Mitochondria were then isolated (at 4 °C) in the presence of 1 mM PMSF. By this method, over 95% of the radiolabeled cytochrome c_1 present in mitochondria was of the intermediate size (see *lane 1*). The mitochondria were then incubated in mixtures (*lanes 1–7*) containing 15 μg of mitochondrial protein in a final volume of 300 μl of 0.25 M sucrose, 10 mM Mops/KOH (pH 7.2), 1 mM PMSF for 30 min at 25 °C (except *lane 1* which remained at 0 °C). The samples also contained NADH, NAD^+, or KCl as indicated. Following the incubation, cytochrome c_1 was immunoprecipitated as described under "Materials and Methods." For *lane 8*, cytochrome c_1 was immunoprecipitated from cells grown continuously in the presence of $^{35}SO_4$ (see "Materials and Methods"). The immunocomplexes were dissociated in SDS-containing sample buffer and resolved on SDS-polyacrylamide gels. The cytochrome c_1 bands from a fluorogram of the resulting gel are shown. The bands were quantified by laser densitometry and the percentage processed to mature size cytochrome c_1 is given (% *m*-C_1). i and m are intermediate and mature size cytochrome c_1, respectively.

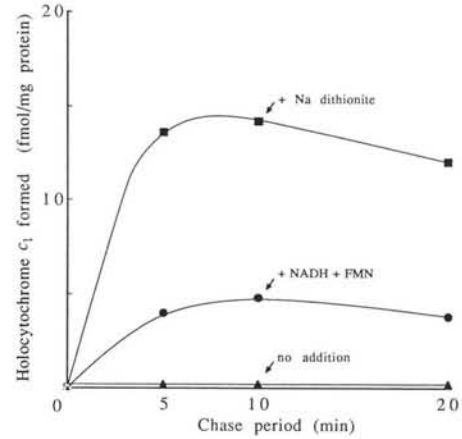


FIG. 8. **Reduction requirements for the chase of accumulated apocytochrome c_1 to holocytochrome c_1 following detergent solubilization.** Intermediate size cytochrome c_1 was accumulated in mitochondria *in vitro* and then chased to holocytochrome c_1 as described for Fig. 7*A*, except that prior to the chase incubation the mitochondria were lysed with octyl glucoside (1% (w/v) final concentration) in the following way: The detergent was added from a 100-fold concentrated stock solution to the mitochondrial preparation (containing accumulated intermediate size cytochrome c_1). The mixture was shaken briefly (10 s) at 1-min intervals for 5 min and then subjected to centrifugation for 20 min at 48,000 × *g*. The resulting supernatant was retained and then chased for varying time periods (as described in Fig. 7*A* for intact mitochondria) without further additions (▲) or in the presence of either 5 mM NADH plus 10 μM FMN (●) or 1 mg of sodium dithionite/ml (■). The holocytochrome c_1 formed was then determined.

tionship between holocytochrome c_1 formation and the ensuing processing to m-C_1.

Heme Attachment in the Presence of Detergent Can Only Be Observed after Apocytochrome c_1 Has First Been Delivered to CC_1HL in Intact Mitochondria—In the case of cytochrome *c* heme lyase, it was possible to solubilize the fully active enzyme from mitochondria with the nonionic detergent octyl glucoside. Such detergent extracts efficiently converted apocytochrome *c* that had been synthesized *in vitro* in reticulocyte lysate to holocytochrome *c*, provided that dithionite was present (the NADH/FMN-mediated reduction of heme was inactive in detergent-lysed mitochondria) (Nicholson and Neupert, 1989).

We have made similar but unsuccessful attempts to solubilize CC_1HL activity from mitochondria using several detergents (including octyl glucoside, lauryl maltoside, CHAPS, and Triton X-100) and using a variety of substrates, including apo(p-C_1) synthesized in reticulocyte lysate, apo(i-C_1) prepared by digestion of p-C_1 with a purified preparation of the matrix processing peptidase, and apo(m-C_1) synthesized in reticulocyte that was directed by transcripts from a plasmid in which the start codon of the presequence had been deleted. On the other hand, when p-C_1 was first imported into intact mitochondria up to the stage where it was processed to i-C_1

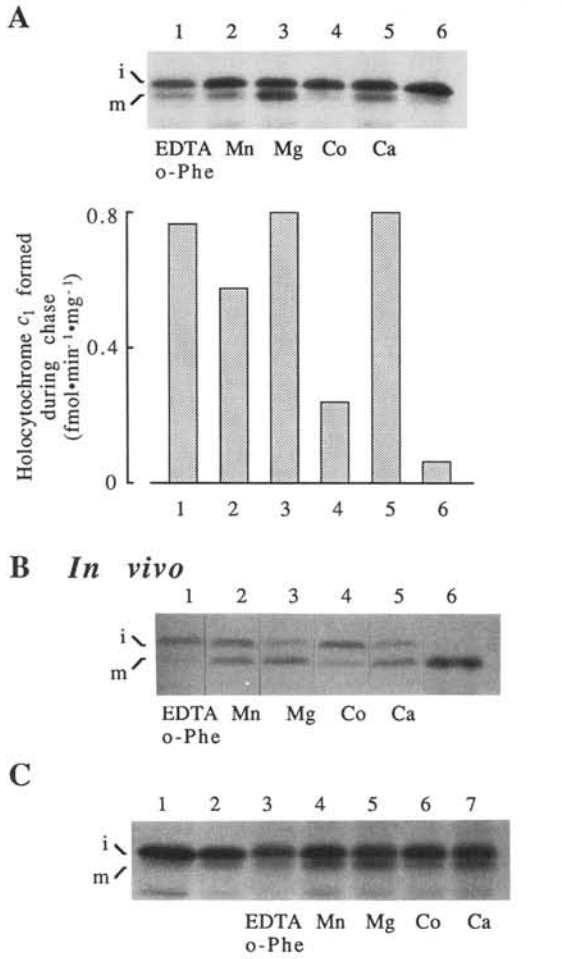


FIG. 9. **Stimulation of second processing of cytochrome c_1 by cations.** Intermediate size cytochrome c_1 was accumulated in mitochondria either *in vitro* (*panels A* and *C*) or *in vivo* (*panel B*) and then chased to mature size cytochrome c_1 (*in vitro* in all cases) in the presence of various additions. *A*, an import pool was prepared containing mitochondria (50 μg/individual sample), [^{35}S]cysteine-labeled cytochrome c_1, and 2.5 mM $MgCl_2$. After incubating for 15 min at 25 °C, the mixture was treated with proteinase K and the mitochondria were reisolated by centrifugation (see "Materials and Methods"). The mitochondrial pellet was resuspended in 0.25 M sucrose, 10 mM Mops/KOH (pH 7.2) and used for subsequent chase reactions. The predominant cytochrome c_1 species that was accumulated was the intermediate size (*lane 6*). Double size mixtures were prepared containing 100 μg of mitochondrial protein (having accumulated intermediate size cytochrome c_1) plus 10 mM FMN and 5 mM NADH (except *sample 6*) in 200 μl of 0.25 M sucrose, 10 mM Mops/KOH (pH 7.2). In addition, the following chelators or cations were present: 5 mM EDTA plus 0.2 mM *o*-phenanthroline (*sample 1*); 5 mM $MnCl_2$ (*sample 2*); 5 mM $MgCl_2$ (*sample 3*); 5 mM $CoCl_2$ (*sample 4*); 5 mM $CaCl_2$ (*sample 5*). The mixtures were incubated for 20 min at 25 °C (except sample 6 which remained at 0 °C), divided, and then the mitochondria were reisolated by centrifugation. The mitochondrial pellets from one aliquot were dissociated in SDS-containing sample buffer and resolved on SDS-polyacrylamide gels. The cytochrome c_1 bands from a fluorogram of the resulting gel are shown (*upper part* of *panel A*). Cytochrome c_1 was immunoprecipitated from the remaining aliquot and the holocytochrome c_1 that was formed during the chase was determined as described under "Materials and Methods" (*lower part* of *panel A*). *B*, radiolabeled intermediate size cytochrome c_1 was accumulated in mitochondria *in vivo* as described for Fig. 7*B*, *lane 1*. The mitochondria were then incubated in mixtures containing mitochondria (15 μg of protein) in a final volume of 300 μl of 0.25 M sucrose, 10 mM Mops/KOH (pH 7.2), 1 mM PMSF, 5 mM NADH (except *lane 6*) plus chelators or divalent cations as described for *panel A*. Following incubation for 20 min at 25 °C, the mitochondria were reisolated by centrifugation, and cytochrome c_1 was immunoprecipitated. The immunocomplexes were dissociated in SDS-containing sample buffer and resolved on SDS-polyacrylamide gels. The cytochrome c_1 bands from a fluorogram of the resulting gel are shown. *C*, an import pool was prepared as described for *panel A* to accumulate the intermediate size cytochrome c_1, except that after proteinase K treatment and centrifugation the mitochondrial pellet was resuspended in 0.25 M sucrose, 10 mM Mops/KOH (pH 7.2) containing 5 mM EDTA and 0.2 mM *o*-phenanthroline (*lane 1*). The cytochrome c_1 accumulated in this way was chased to holocytochrome c_1 by adding hemin (3 μM final concentration) and sodium dithionite (1 mg/ml) and incubating the pool for 10 min at 25 °C in a reaction mixture that contained 50 μg of mitochondrial protein/200 μl of the buffer described above. The mitochondria were again reisolated by centrifugation and suspended in 0.25 M sucrose, 10 mM Mops/KOH (pH 7.2) (*lane 2*). Approximately 65% of the cytochrome c_1 present was the heme-containing (holo) form (not shown). The samples were chased a second time for 20 min at 25 °C in mixtures containg 50 μg of mitochondrial protein and 25 μM deuterohemin (to block further holocytochrome c_1 formation) in a final volume of 200 μl of the sucrose/Mops buffer (see above) plus 5 mM EDTA and 0.2 mM *o*-phenanthroline (*lane 3*), 5 mM $MnCl_2$ (*lane 4*), 5 mM $MgCl_2$ (*lane 5*), 5 mM $CoCl_2$ (*lane 6*), or 5 mM $CaCl_2$ (*lane 7*). The samples were then processed as described in *panel A* for gel electrophoresis and fluorography. *i* and *m* are intermediate and mature size cytochrome c_1, respectively. *o-phe*, *o*-phenanthroline.

and formed a salt-resistant complex with CC_1HL (stage IV in Fig. 6), the complex could be subsequently solubilized with octyl glucoside and the apocytochrome c_1 could then be efficiently converted to holocytochrome c_1 (Fig. 8). Unlike the reaction in intact mitochondria, holocytochrome c_1 formation was poor in the presence of NADH and FMN, but it did occur with comparable efficiency when dithionite was used as a reductant (*cf.* Fig. 7*A*). Moreover, the stages of import at which holocytochrome c_1 formation could occur in detergent-solubilized preparations of mitochondria corresponded identically to the stages at which apocytochrome c_1 formed a salt-resistant complex with CC_1HL (Table I).

We suggest that the introduction of apocytochrome c_1 to CC_1HL along a specific delivery route is necessary before it becomes a competent substrate for holocytochrome c_1 formation. Solubilization of mitochondria with detergents may disrupt this "introductory" pathway. One possibility is that apocytochrome c_1 must approach CC_1HL in a specific orientation, perhaps as it is retranslocated back across the inner membrane, in order for it to be correctly recognized by CC_1HL. Another possibility is that conformational changes in the cytochrome c_1 polypeptide occur during the earlier stages of its import pathway which are necessary for it to become a competent substrate for conversion to holocytochrome c_1.

The Second Proteolytic Processing Step of Cytochrome c_1 Import Is Stimulated by Cations—The matrix processing peptidase-catalyzed processing of p-C_1 to i-C_1 is strongly dependent on divalent cations. In order to examine the cation requirements of the second proteolytic processing step, a specific assay had to be developed. Cytochrome c_1 was imported and accumulated as i-C_1 in the presence of $MgCl_2$, but absence of NADH (Fig. 9*A*). The mitochondria were treated with proteinase K, reisolated, and the accumulated i-C_1 (*lane 6*) was chased in the presence of NADH, FMN, and various divalent cations as indicated (*lanes 1–5*). In three cases (samples 1, 3, and 5) the amounts of holocytochrome c_1 formed during the chase incubation were nearly the same (*lower half*, Fig. 9*A*). Processing to m-C_1 among these three samples, on the other hand, was clearly stimulated by the presence of $MgCl_2$ (*lane 3*) and to a lesser degree by $CaCl_2$ (*lane 5*). This then suggests that given a constant amount of holocytochrome c_1 available, processing from holo(i-C_1) to holo(m-C_1) was stimulated by specific divalent cations ($Mg^{2+} > Ca^{2+}$). The same stimulation was also observed for the processing of i-C_1 that had been

accumulated *in vivo* to m-C_1 (Fig. 9*B*).

Another approach was taken to determine whether this effect was specific for proteolytic processing in the absence of ongoing holocytochrome c_1 formation (Fig. 9*C*). Intermediate size cytochrome c_1 was first accumulated in mitochondria in the presence of $MgCl_2$ and absence of reducing agents (*lane 1*) as described for Fig. 9*A*. The accumulated i-C_1 was then chased in a second incubation to holocytochrome c_1 in the presence of EDTA/*o*-phenanthroline and dithionite. Approximately 65% of the cytochrome c_1 present was converted to holocytochrome c_1, but only a small proportion was processed to m-C_1 (*lane 2*). The mitochondria were washed, reisolated, and treated with deuterohemin to block further holocytochrome c_1 formation. Processing to m-C_1 was then examined in a third incubation in the presence or absence of divalent cations (*lanes 3–8*). Processing to m-C_1 was stimulated by divalent cations ($Mg^{2+} > Ca^{2+} > Mn^{2+} > Co^{2+}$) as well as by monovalent cations (80 mM KCl or NaCl, not shown), whereas some degradation of the partially imported i-C_1 occurred in the sample containing EDTA and *o*-phenanthroline (*lane 3*).

It therefore appears that the processing of i-C_1 to m-C_1 (subsequent to holocytochrome c_1 formation) is stimulated by the presence of divalent and monovalent cations. Furthermore, the reaction shows some specificity since in all three approaches Mg^{2+} was the most effective cation.

DISCUSSION

Processing of cytochrome c_1 during import into mitochondria appears to occur by the following sequence of reactions. (i) During or following the import of p-C_1 into the mitochondrial matrix, the "matrix-targeting" domain of the bipartite presequence is removed by matrix processing peptidase. (ii) The i-C_1 thus formed is re-exported to the intermembrane-space side of the inner membrane where heme is covalently linked to it by the enzyme cytochrome c_1 heme lyase. (iii) A second processing peptidase then removes the remainder of the presequence (the "re-export" domain, see below) thereby generating m-C_1 that is ready for assembly into the cytochrome bc_1 complex.

Properties and Roles of Processing Steps during Cytochrome c_1 Biogenesis—The covalent attachment of heme to apocytochrome c_1 occurs on the outer face of the inner mitochondrial membrane and appears to require that the heme be in the reduced state. Both p-C_1 and i-C_1 can be accepted as substrates for this reaction; however, the apocytochrome c_1 must be delivered to CC_1HL in a highly specific manner, a process that does not occur after disruption of mitochondria with detergents. The CC_1HL enzyme itself is membrane-associated and probably faces the intermembrane space. It is clearly distinct from the equivalent enzyme that catalyzes heme addition to cytochrome *c* during import (namely, cytochrome *c* heme lyase) (Dumont *et al.*, 1987; Nargang *et al.*, 1988).

The bipartite presequence of cytochrome c_1 is removed in two steps, the first being catalyzed by matrix processing peptidase in the mitochondrial matrix. Removal of the matrix-targeting domain of the presequence is not, however, absolutely required for retranslocation of cytochrome c_1 from the matrix to the intermembrane space, although it does enhance the efficiency of export from the matrix. This situation is similar to that observed for the export of proteins from the bacterial cytoplasm and for the translocation of proteins across the membrane of the endoplasmic reticulum. For example, the signal sequence of M13 procoat protein when internalized within a fusion protein (constructed by joining the amino-terminal 141 residues of cytoplasmic ribulokinase to the amino terminus of all but the first 10 residues of M13 procoat) was able to direct the fusion protein to the plasma membrane where it was processed by leader peptidase giving rise to a leader peptide of 155 residues and mature coat protein (Kuhn, 1987). Similarly, a hybrid protein containing globin (first 109 amino acids) in front of the precursor to prolactin was found to be completely translocated across microsomal membranes (Perara and Lingappa, 1985). Therefore, the signal responsible for the export of cytochrome c_1 out of the mitochondrial matrix is still functional even when another polypeptide sequence is present at the amino terminus.

The second proteolytic processing step during cytochrome c_1 import occurs on the outer face of the inner membrane following transport from the matrix and heme linkage. Cleavage to generate m-C_1 can occur using either p-C_1 or i-C_1 as a substrate, resulting in single step removal of the bipartite presequence in the former case. Under all circumstances, however, second proteolytic processing occurs only after covalent heme attachment has taken place. In addition to being dependent on the presence of the heme group, the second proteolytic processing step has specific cation requirements.

What then are the roles of the individual processing steps during the import of cytochrome c_1 into mitochondria? We suggest the following possibilities. The cytochrome c_1 presequence is a bipartite structure. The amino-terminal part of its comprises the matrix-targeting domain which directs p-C_1 into the mitochondrial matrix (van Loon *et al.*, 1986). Once in the matrix, cytochrome c_1 can enter its "ancestral" assembly pathway for further sorting and processing (Hartl *et al.*, 1986, 1987). The remainder of the presequence, therefore, is probably responsible for the export of cytochrome c_1 from the matrix to the intermembrane space along the conserved part of its assembly pathway. This is strongly supported by the finding that a hybrid protein consisting of the complete bipartite presequence of cytochrome c_1 and of cytochrome *c* as a passenger protein can be imported into the mitochondrial matrix.[4] In addition, there is remarkable sequence similarity between the second part of the mitochondrial cytochrome c_1 presequence and the export signal sequences of bacterial cytochromes c_1 and c_2. Even though export from the mitochondrial matrix can occur when the entire cytochrome c_1 presequence is present, the role of matrix processing peptidase during assembly is apparently to remove the matrix-targeting information and thereby enhance the efficiency of export from the matrix. For the most part, the role of heme addition is clear. It is required for the functional properties of cytochrome c_1 as an electron carrier, although the possibility that heme linkage is involved in other assembly processes cannot be excluded (see below). Why covalent heme attachment must precede the second proteolytic processing step is not so obvious. One possibility is that cytochrome c_1 is delivered to CC_1HL as it is transported across the inner membrane from the matrix and is simply not accessible to the second processing peptidase until it is released by CC_1HL following heme addition. Another possibility is that the second processing peptidase requires a specific conformation around the cleavage site that only exists within the holo-form of cytochrome c_1. This may safeguard newly imported cytochrome c_1 from premature processing to m-C_1 that might otherwise allow it to enter subsequent assembly steps without a functional ligand.

Divergent Evolution of Import Pathways for Mitochondrial c-type Cytochromes—Despite a number of functional similarities, the import pathways for the two mitochondrial *c*-type cytochromes (namely, cytochromes *c* and c_1) are strikingly

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

different. Both proteins are located in the mitochondrial intermembrane space (cytochrome c_1 being anchored to the inner membrane) and function at the outer surface of the inner membrane; both are involved in mediating the transport of electrons; and both proteins contain heme that is covalently linked by thioether bridges to a highly conserved heme binding site within the respective polypeptide chains. Whereas the import of cytochrome c_1 into mitochondria is complex, however, the import of cytochrome *c* follows an entirely different pathway that is comparatively simple. The precursor (apocytochrome *c*) does not contain a removable targeting prepiece (Zimmermann *et al.*, 1979; Stewart *et al.*, 1971; Zitomer and Hall, 1976; Smith *et al.*, 1979; Matsurra *et al.*, 1981; Scarpulla *et al.*, 1981; Stuart *et al.*, 1987) and differs from its mature counterpart only by the absence of covalently bound heme and by a less folded conformation (Fisher *et al.*, 1973; Rietveld *et al.*, 1985). Apocytochrome *c* can spontaneously penetrate part way through the lipid bilayer of the mitochondrial membrane system (Rietveld and de Kruijff, 1984; Dumont and Richards, 1984; Rietveld *et al.*, 1985, 1986; Berkhout *et al.*, 1987) after which it is bound with high affinity to specific binding sites (Hennig and Neupert, 1981; Hennig *et al.*, 1983; Nicholson *et al.*, 1988b). Import from these binding sites is independent of a membrane potential (Zimmermann *et al.*, 1981; Nicholson *et al.*, 1988a). Instead, complete translocation across the outer membrane is closely coupled to covalent heme addition by the enzyme cytochrome *c* heme lyase, which faces the intermembrane space (Nicholson *et al.*, 1988a, 1988b). A likely mechanism is that the conformational change resulting in the attachment of heme to apocytochrome *c* and the ensuing folding of the cytochrome *c* polypeptide around the heme group drives the transport across the outer membrane into the intermembrane space (Hennig and Neupert, 1981; Nicholson *et al.*, 1988b). In this process, the transmembrane movement of cytochrome *c* into mitochondria is mechanistically coupled to the heme attaching event. This is in marked contrast to the situation for cytochrome c_1 where the precursor protein is imported and sorted to its final submitochondrial location before heme attachment takes place (although the possibility that a conformational change in the cytochrome c_1 polypeptide following heme linkage aids in the completion of transport of what might otherwise be an only partially translocated molecule cannot be excluded).

Even though the mitochondrial import pathways for cytochromes *c* and c_1 are different, they have apparently evolved from a common starting point. For example, in photosynthetic bacteria both the equivalent of mitochondrial cytochrome *c* (*i.e.* cytochrome c_2 from *Rhodopseudomonas capsulata*) and of cytochrome c_1 (*i.e.* cytochrome c_1 from *Rhodopseudomonas sphaeroides*) are synthesized in the bacterial cytoplasm (comparable to the mitochondrial matrix) with an amino-terminal export leader sequence (Daldal *et al.*, 1986; Gabellini and Sebald, 1986). Transport across the photosynthetic membrane to the side opposite the F_1 part of ATPase (comparable to transport across the inner mitochondrial membrane to the intermembrane space) is accompanied by proteolytic processing to yield the mature size protein (Gabellini *et al.*, 1985). We propose that during evolution the respective assembly pathways for cytochromes *c* and c_1 have diverged. Whereas cytochrome c_1 import into mitochondria has retained remnants of its ancestral assembly pathway, cytochrome *c* import occurs by a simpler and novel mechanism. Cytochrome *c* has probably escaped the conservative sorting that cytochrome c_1 must follow by taking advantage of its membrane active nature, which allows entry into the outer membrane without the help of surface receptors, and by the coupling of heme linkage to membrane translocation.

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