

Cytochrome Systems

**Molecular Biology
and Bioenergetics**

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**NADH: A COMMON REQUIREMENT FOR THE IMPORT
AND MATURATION OF CYTOCHROMES c AND c₁**

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SUMMARY

The covalent attachment of heme to apocytochrome c, which is catalyzed by the mitochondrial enzyme cytochrome c heme lyase, was dependent on NADH. In addition, a cofactor present in reticulocyte lysate or a *Neurospora crassa* cytosol fraction was required for the NADH-dependent step. In the absence of NADH, apocytochrome c was bound to the mitochondrial surface and remained accessible to externally added proteases. In the presence of NADH, covalent attachment of heme occurred with concomitant translocation of cytochrome c across the outer mitochondrial membrane to a protease-resistant location. Both heme attachment and translocation were inhibited by the heme analogue deuterohemin.

The second proteolytic-processing step during cytochrome c₁ import, from the intermediate to mature-size protein, was also dependent on NADH and could also be inhibited by deuterohemin. We suggest that this occurs as a result of conditions which affect the heme-attaching reaction for cytochrome c₁ and that heme attachment must precede the second processing step. Models are presented to account for these observations.

INTRODUCTION

The majority of mitochondrial proteins are encoded by nuclear genes and are synthesized as precursors on free ribosomes in the cell cytosol. They are then imported into mitochondria along pathways which can be subdivided into a consecutive series of distinct steps (for review see 1-4). Most mitochondrial proteins, particularly those which must be directed to the inner membrane or matrix, are imported by the following sequence of events: i) synthesis in the cytosol as a precursor containing an N-terminal targeting sequence; ii) binding to specific receptors on the mitochondrial surface; iii) membrane-potential-dependent insertion into the inner membrane via translocation contact sites where the inner and outer membrane come close enough together to be spanned simultaneously by the imported protein; iv) removal of the N-terminal prepiece by the chelator-sensitive processing peptidase located in the matrix; v) sorting of the protein to its final sub-mitochondrial location; and vi) assembly into functional complexes. In a number of cases, the import pathway is accompanied by covalent modification

or the acquisition of non-covalently bound prosthetic groups.

Energy is required for protein import in three known forms. First, it has been well established that all proteins which must be translocated into or across the inner membrane require the presence of a membrane potential (5,6). Recently, it has also been demonstrated that ATP is required for the maintenance of an import-competent conformation during all steps which precede and include interaction of the precursor protein with the outer membrane (7, see accompanying article by Hartl et al). Finally, NAD(P)H is required for the maturation of cytochromes c (8,9) and c_1 (10,11), for the Fe/S protein of the bc_1 complex (Hartl and Neupert, unpublished), and for cytochrome oxidase subunit II (Driever, Cook and Neupert, unpublished).

The requirement for NADH by cytochromes c and c_1 appears to be a common feature of the later stages of their respective import pathways. Despite a number of similarities between the two proteins (e.g. both mature proteins are exposed to the mitochondrial intermembrane space; they are the only two mitochondrial proteins to which heme is covalently bound; they both require NADH during import), cytochromes c and c_1 follow markedly different import pathways. Cytochrome c is synthesized in the cytosol as apocytochrome c, which differs from its mature counterpart in conformation and by the absence of covalently-bound heme. It does not contain a cleavable N-terminal prepiece and does not require a membrane potential for import (12-18). Apocytochrome c is transferred from a cytoplasmic pool to specific receptors at the outer mitochondrial membrane (18-21). Heme is attached to the apoprotein, by the enzyme cytochrome c heme lyase (8,9,20,22-24), in a step which is coupled to the translocation of cytochrome c across the outer membrane to its final location in the intermembrane space. Cytochrome c_1 , on the other hand, follows a somewhat more complicated sequence of events during import. The precursor of cytochrome c_1 is synthesized in the cytosol and contains an unusually long N-terminal prepiece which is processed in two steps (10,11,25,26). The precursor binds to receptors at the outer membrane and the N-terminus is then translocated through the inner membrane via contact sites (11) in a step which is dependent on a membrane potential. The hydrophilic first half of the prepiece is removed by the matrix peptidase followed by removal of the second part by another processing activity which is presumed to be located in the intermembrane space. The latter step is dependent on heme (25,26) and therefore might be coupled to the heme attaching reaction or might have to be preceded by heme linkage. The mature protein is then assembled into the bc_1 complex, anchored to the inner membrane by a hydrophobic stretch of amino acids at the C-terminus (27,28).

In this report we have examined the common requirement for NADH during the import of cytochromes c and c_1 into mitochondria. We demonstrate that NADH is required for the covalent attachment of heme to apocytochrome c and that the NADH-dependent step is mediated by a cytosolic cofactor. Since the second processing step of cytochrome c_1 has nearly identical requirements, we suggest that NADH is needed for a similar import event; namely, for the activity of cytochrome c_1 heme lyase.

MATERIALS AND METHODS

Cell Growth and Subcellular Fractionation

Neurospora crassa wild type 74A was grown for 14 h at 25°C as previously described (29). Mitochondria were isolated from freshly harvested hyphae by Percoll-gradient centrifugation (Pharmacia) and suspended in buffer A (250 mM sucrose, 10 mM Mops/KOH (pH 7.2), 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF)) at a protein concentration of 5 mg/ml (8). PMSF was not included in buffer A during any of the fractionation or import steps if

proteinase K digestion was included in the experimental procedure. A cytosol fraction was prepared by grinding hyphae with quartz sand in buffer A and then preparing a post-ribosomal supernatant (8).

Cell-Free Protein Synthesis

Precursor proteins of *N. crassa* cytochromes c and c_1 were synthesized in rabbit reticulocyte lysates (30) in the presence of L-(35 S)cysteine as previously described (8). Synthesis of apocytochrome c was directed by *N. crassa* poly(A)-containing RNA (31). Synthesis of the precursor to cytochrome c_1 was directed by a full-length cDNA clone of pre-cytochrome c_1 in pGEM-4 that was transcribed with SP6 RNA-polymerase (Ostermann and Neupert, unpublished).

Import of Cytochrome c

1. Direct import: Mitochondria (75 μ g protein) were incubated for 10 min at 25°C in a total volume of 200 μ l buffer A in the presence of 50 μ l (35 S)cysteine-labelled reticulocyte lysate plus other additions as indicated. Holocytochrome c formation was determined by re-isolating the mitochondria by centrifugation, immunoprecipitating total cytochrome c (holo plus apo), dissociation of the immunocomplexes with urea, digestion with trypsin, and analysis of the resulting peptides by reverse-phase HPLC (8). The holocytochrome c cysteine-containing tryptic peptide was collected and its radioactivity determined as a measure of holocytochrome c formation.

2. Chase of pre-bound apocytochrome c to holocytochrome c: Apocytochrome c was bound to mitochondria in the absence of conversion to holocytochrome c (by withholding NADH) by incubating mitochondria (75 μ g protein) with 50 μ l (35 S)cysteine-labelled reticulocyte lysate in a total volume of 200 μ l buffer A for 10 min at 25°C. The mitochondria were re-isolated by centrifugation at 17,400 xg for 12 min and resuspended in fresh buffer A. The bound apocytochrome c was then chased to holocytochrome c during a second incubation for 10 min at 25°C in a mixture containing 75 μ g mitochondrial protein, plus additions as indicated, in 200 μ l buffer A. Holocytochrome c formation was then determined as described above.

Import of Cytochrome c_1

1. Direct import: Mitochondria (50 μ g) were incubated for 30 min at 25°C in a mixture (total volume 100 μ l) containing 3% (w/v) bovine serum albumin, 70 mM KCl, 220 mM sucrose, 10 mM Mops/KOH (pH 7.2) in the presence of 20 μ l (35 S)cysteine-labelled reticulocyte plus other additions as indicated. The samples were cooled to 0°C and proteinase K was added to a final concentration of 20 μ g/ml. Following incubation for 30 min at 0°C, the mitochondria were re-isolated as above and dissociated in SDS sample buffer. The samples were then resolved by SDS polyacrylamide gel electrophoresis and visualized by fluorography of the dried gel.

2. Chase of intermediate to mature-size cytochrome c_1 : The precursor was imported into mitochondria as above but in the presence of 2.5 mM $MgCl_2$ and absence of NADH to accumulate the intermediate-size protein. The mixtures were then treated with proteinase K (Fig. 4D) or not (Fig 4C) as described above. The mitochondria were re-isolated and suspended in buffer A then incubated for 30 min at 25°C in the presence of additions, as indicated, in a total volume of 100 μ l. The samples were diluted with 1 ml buffer A and the mitochondria were sedimented by centrifugation and then dissociated in SDS sample buffer as above.

Miscellaneous Methods

Cytochrome c was purified from *N. crassa* and specific antibodies were raised in rabbits as previously described (29). Radioactivity determinations

were performed by scintillation counting in 0.1 ml of 10% (w/v) SDS, 1 M Tris/HCl (pH 8.0), plus 10 ml ACS II (Amersham). SDS polyacrylamide gel electrophoresis was performed using standard techniques (32). Gels were prepared for fluorography by soaking them for 30 min in Amplify (Amersham) followed by drying and then exposure to X-ray films at -80°C . The bands were quantified by laser densitometry (LKB-GSXL). Protein was determined as described previously (33).

RESULTS

Covalent Attachment of Heme to Apocytochrome c Requires NADH and a Cofactor from Reticulocyte Lysate or Cell Cytosol

In the absence of reducing agents, the import of cytochrome c into mitochondria and formation of holocytochrome c occurred at only very low rates. In the presence of NADH, however, holocytochrome c formation was stimulated 8 to 12-fold (figure 1). The NADH-dependent activity of cytochrome c heme lyase was not affected by the presence of valinomycin/ K^+ (which dissipates the membrane potential) or by antimycin A/oligomycin (which blocks the generation of a membrane potential). Other reducing agents could substitute for the NADH requirement to varying degrees. NAD^+ alone stimulated activity to 22% (at 2 mM) of the optimum observed with NADH (at 5 mM). In the presence of an NADH-regenerating system (L(+)lactate plus lactate dehydrogenase), NAD^+ behaved essentially the same as NADH.

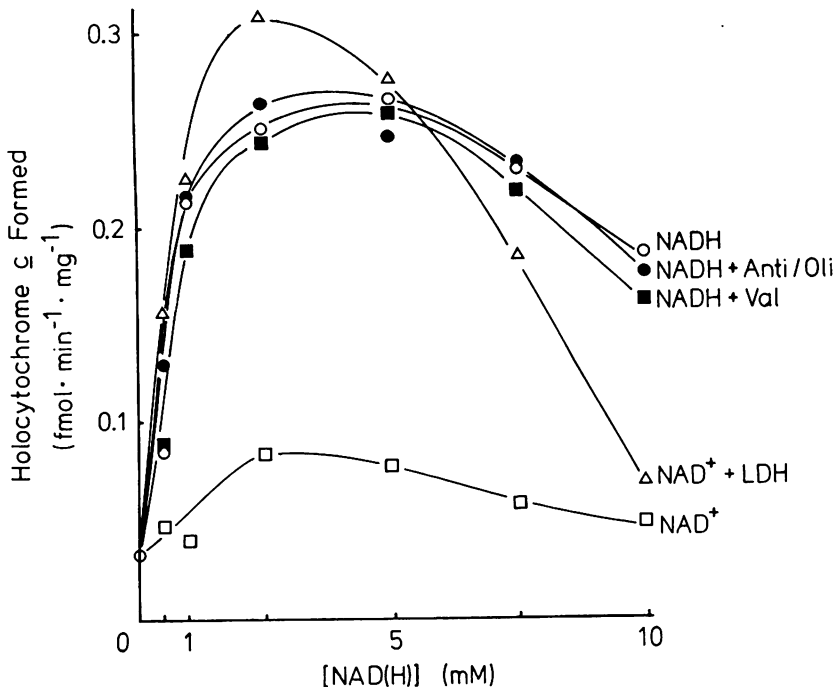


Fig. 1 Holocytochrome c Formation Depends on NADH. Apocytochrome c, synthesized in reticulocyte lysate, was imported into mitochondria in the presence of varying amounts of either NAD^+ (\square, \triangle) or NADH ($\circ, \bullet, \blacksquare$) plus other additions as follows: \circ, \square , no further additions; \triangle , 45 mM L(+)lactate, 3 U lactate dehydrogenase (EC 1.1.1.27) per ml; \bullet , 8 μM antimycin A, 20 μM oligomycin; \blacksquare , 0.5 μM valinomycin. Holocytochrome c formation was determined following incubation for 10 min at 25°C .

NADPH (at 1 mM), which has been reported to be required for holocytochrome c formation in yeast (9), stimulated activity to 45% of the NADH optimum (8), as did glutathione (43% at 25 mM)(8), Na dithionite (100% at 1 mg/ml, unpublished), and 2-mercaptoethanol (7% at 50 mM, unpublished).

We have previously investigated the role of NADH in the covalent attachment of heme to apocytochrome c (8). NADH was not required for the binding of apocytochrome c to mitochondria and, unlike the majority of imported mitochondrial proteins, a membrane potential was not necessary for any of the stages of cytochrome c import. NADH was not involved in the reduction of the cysteine thiols to which heme is covalently attached. Preliminary experiments indicate that reduction of the heme iron from Fe(III) to Fe(II) must occur before the enzymatic linkage of heme to apocytochrome c, and that this reduction step requires NADH (Nicholson and Neupert, in preparation).

Apocytochrome c could be bound to mitochondria in the absence of conversion to holocytochrome c by not including NADH in the incubation mixtures. When the mitochondria were re-isolated and washed, to remove constituents from the reticulocyte lysate, the bound apocytochrome c was inefficiently chased to holocytochrome c after adding NADH unless the mixtures were supplemented with either unlabelled reticulocyte lysate or a cell cytosol fraction (figure 2). Neither the reticulocyte lysate or cytosol fraction had any stimulatory activity in the absence of NADH, and there was no measurable cytochrome c heme lyase activity in these preparations alone. The cofactor was a heat-stable, protease-insensitive, dialyzable component which could presumably reach the intermembrane space, where heme attachment occurs, via the channels formed by porin (8).

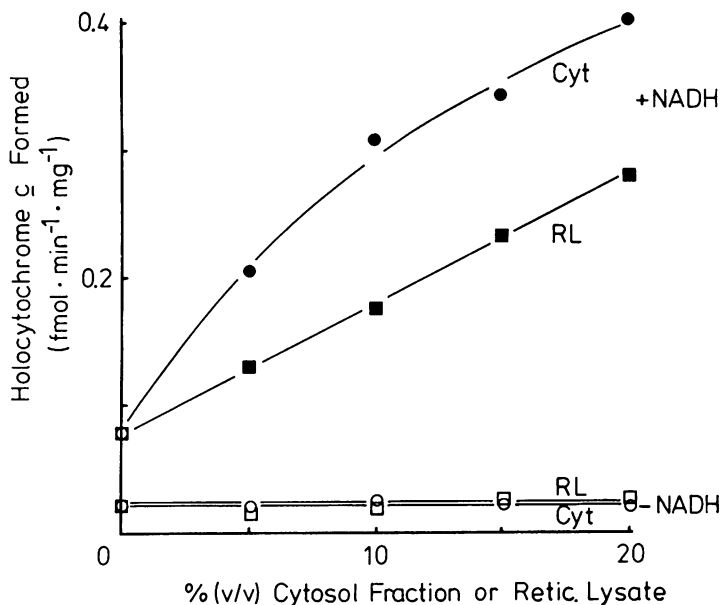


Fig. 2 NADH-Dependent Holocytochrome c Formation Depends on a Cofactor. Apocytochrome c, synthesized in reticulocyte lysate, was pre-bound to mitochondria in the absence of NADH (to prevent conversion to holocytochrome c). The mitochondria were reisolated and the bound apocytochrome c was chased to holocytochrome c for 10 min at 25°C in the presence (●, ■) or absence (○, □) of 5 mM NADH, plus varying amounts of either cytosol fraction (●, ○) or unlabelled reticulocyte lysate (■, □). Holocytochrome c formation was then determined.

Covalent attachment of heme to apocytochrome c, by the enzyme cytochrome c heme lyase, requires NADH in a step which is also dependent on a factor present in reticulocyte lysate or *N. crassa* cell cytosol. This step appears to be the reduction of the heme iron and the cofactor probably mediates this reduction. The conformation of the heme group and/or its ability to form critical alignments with apocytochrome c may be essential for the cytochrome c heme lyase reaction.

The Translocation of Cytochrome c to a Protease-Resistant Location Depends on NADH and is Inhibited by Deuterohemin

We have previously demonstrated that the apocytochrome c associated with mitochondria in the presence of deuterohemin (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) could be displaced by excess added apocytochrome c; e.g. import was blocked at the stage of high-affinity receptor binding (20). We therefore proposed that the translocation of cytochrome c across the outer mitochondrial membrane during import is intrinsically coupled to the covalent attachment of heme (8,20).

Translocation of cytochrome c across the outer mitochondrial membrane was determined by the accessibility of the protein to externally added proteases which did not penetrate the outer membrane under the conditions used here (figure 3). In the presence of NADH, formation of holocytochrome c occurred at 23-times the rate compared to samples which did not contain NADH (mixtures II and I, respectively). Translocation of the cytochrome c to a protease-resistant location also depended on NADH (cf. lane 1 vs 3). As a control, the imported cytochrome c was sensitive to protease treatment when the mitochondria were lysed with detergent following import (lanes 2 and 4).

In the presence of NADH, 80% of the conversion of apocytochrome c to holocytochrome c was inhibited by deuterohemin (cf. mixture III vs IV). In an identical manner, the translocation of cytochrome c to a protease-resistant location was inhibited by approximately 80% in the presence of deuterohemin (cf. lane 5 vs 7). Therefore, whenever holocytochrome c formation was stimulated by NADH, or inhibited by deuterohemin, there were parallel changes in the formation of a protease-resistant cytochrome c species.

The close correlation between the formation of holocytochrome c and the transport of the protein to a location in mitochondria where it was not accessible to externally added proteases confirms our hypothesis that the two events are tightly coupled.

Formation of Mature-Size Cytochrome c_1 Depends on NADH and is Inhibited by Deuterohemin

The import of cytochrome c_1 could be arrested at different stages under various conditions (figure 4A).¹ When import was performed in the presence of EDTA/o-phenanthroline, which inhibits the matrix peptidase, the precursor of cytochrome c_1 (p- C_1) was accumulated in a protease-resistant location (lane 1). In the presence of $MgCl_2$, the imported cytochrome c_1 was processed to the intermediate-size protein (i- C_1 ; lane 2). Processing to the mature-size cytochrome c_1 (m- C_1) depended on the presence of NADH in addition to $MgCl_2$ (lane 3). The second processing step, or events tightly coupled to the second processing step (e.g. covalent attachment of heme), therefore appear to require NADH.

To determine whether the second processing step of cytochrome c_1 maturation is coupled to heme linkage, cytochrome c_1 was imported in the presence or absence of deuterohemin (figure 4B). In the absence of NADH,

cytochrome c_1 accumulated as the intermediate-size protein with negligible formation of $m-C_1$ (lane 1). In the presence of NADH, more than 65% of the imported cytochrome c_1 appeared as $m-C_1$ (lane 2). When deuterohemin was included in import mixtures that were otherwise identical to that in lane 2, formation of $m-C_1$ was inhibited (lane 3). Formation of $m-C_1$ in the presence of deuterohemin occurred at 39% of the rate compared to that in the absence of deuterohemin (cf. lane 2 vs 3). Although the total amount of imported cytochrome c_1 was lower in the presence of deuterohemin, the ratio of $m-C_1$ to $i-C_1$ in the presence of deuterohemin (0.66) was considerably lower than the ratio in the absence of deuterohemin (2.1) indicating that the second processing step was specifically blocked.

Similar results were observed when cytochrome c_1 was imported from reticulocyte lysate into mitochondria in the absence of NADH, then the mitochondria were reisolated and the bound cytochrome c_1 was chased under various conditions (figure 4C). Low amounts of the bound cytochrome c_1 were chased to $m-C_1$ in the absence of NADH (lane 1). In the presence of NADH, however, $m-C_1$ formation was stimulated 3.2-fold (lane 2). This NADH-dependent chase to $m-C_1$ was blocked when deuterohemin was added to the incubation mixture (lane 3). When unlabelled reticulocyte lysate was added during the chase incubation, the NADH-dependent $m-C_1$ formation was stimulated a further 39% (cf. lane 4 vs 2). Processing to $m-C_1$ under these conditions

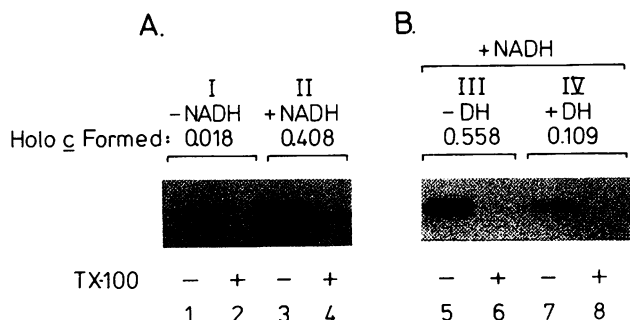


Fig. 3 **A.** Translocation of Cytochrome c to a Protease-Resistant Location Depends on NADH. Apocytochrome c , synthesized in reticulocyte lysate, was imported into mitochondria in the absence (pool I) or presence (pool II) of 5 mM NADH. Aliquots of the two samples were removed for determination of holocytochrome c formation (expressed as $\text{fmol} \times \text{min}^{-1} \times \text{mg}^{-1}$). The samples were then divided into two aliquots, one of which received Triton X-100 (1% (w/v) final concentration) (lanes 2 & 4). All four samples were treated with 40 μg proteinase K/ml for 25 min at 25°C at a mitochondrial protein concentration of 1 mg/ml. (Using this procedure, less than 5% of adenylate kinase activity, a marker for the intermembrane space, was lost in the absence of Triton X-100 while more than 90% was lost in the presence of detergent.) Total cytochrome c was immunoprecipitated from the samples, resolved by SDS-PAGE, and visualized by fluorography. **B.** Translocation of Cytochrome c to a Protease-Resistant Location is Inhibited by Deuterohemin. Apocytochrome c was imported into mitochondria in mixtures containing 5 mM NADH and in either the absence (pool III) or presence (pool IV) of 100 μM deuterohemin. Holocytochrome c formation and translocation to a protease-resistant location were then determined as described for panel A.

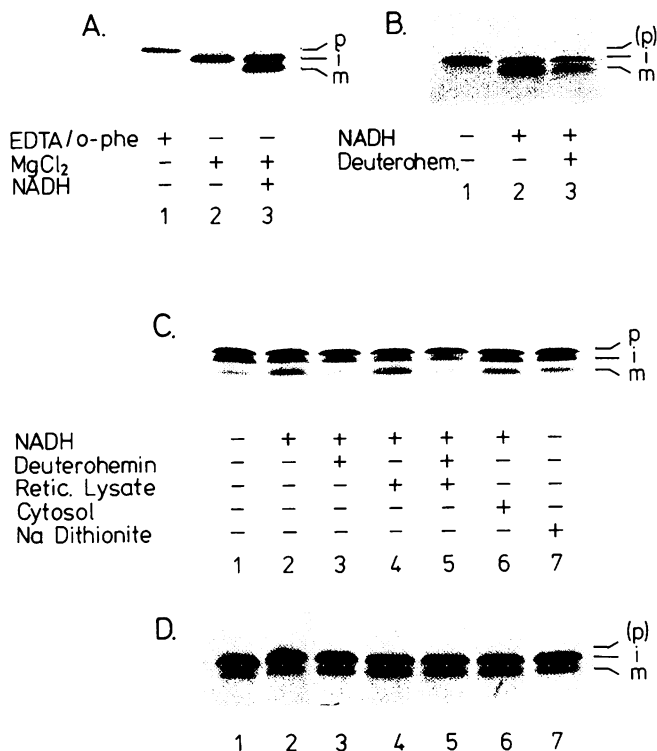


Fig. 4 **A.** Import of Cytochrome c_1 into Mitochondria. The precursor of cytochrome c_1 was imported into mitochondria in the presence of either 5 mM EDTA plus 0.2 mM o-phenanthroline, 2.5 mM MgCl₂, or 5 mM NADH as indicated. Following incubation for 30 min at 25°C, the samples were digested with proteinase K for 30 min at 0°C and then the mitochondria were reisolated, dissociated in SDS sample buffer and processed as described in Methods. p, precursor; i, intermediate; m, mature. **B.** The NADH-Dependent Second Processing Step of Cytochrome c_1 is Inhibited by Deuterohemin. The precursor of cytochrome c_1 was imported into mitochondria, as described in panel A, in the presence of 2.5 mM MgCl₂, plus 5 mM NADH or 0.1 mM deuterohemin as indicated. **C.** Chase of Mitochondrial-Bound Cytochrome c_1 . The precursor of cytochrome c_1 was imported into mitochondria in the presence of 2.5 mM MgCl₂ for 30 min at 25°C. The mitochondria were re-isolated by centrifugation and suspended in buffer A. The bound cytochrome c_1 was chased for 30 min at 25°C in buffer A alone or in the presence of 5 mM NADH, 0.1 mM deuterohemin, 25% (v/v) unlabelled reticulocyte lysate, 20% (v/v) cytosol fraction, or 1 mg Na dithionite/ml as indicated. Mitochondria were reisolated and analyzed as above. **D.** Chase of Protease-Resistant Intermediate-Size Cytochrome c_1 . The experiment was performed as described for panel C except that following the first incubation period the samples were treated with proteinase K as described in panel A.

was also inhibited by deuterohemin (lane 5). The additional stimulation of m-C₁ formation observed in the presence of reticulocyte lysate (lane 4) occurred only marginally (about 5%) when a cell cytosol fraction was used instead (lane 6). Like the NADH-requiring step of cytochrome c import, Na dithionite could be used as a reducing agent for m-C₁ formation with comparable efficiency (cf. lane 7 vs 2).

A similar experiment as described in figure 4C was attempted to examine only the chase from i-C₁ to m-C₁. The intermediate-size cytochrome c₁ was accumulated by importing p-C₁ into mitochondria in the absence of NADH followed by treatment with proteinase K to remove p-C₁ that might be bound to the mitochondrial surface. Under these conditions the mitochondria contained mostly i-C₁ and very little protease resistant p-C₁ (figure 4A, lane 2). The mitochondria were then re-isolated and i-C₁ was chased to m-C₁ under various conditions (figure 4D). Although the efficiency of the chase was low following this treatment, the same basic features described in figure 4C were observed, namely: m-C₁ formation was stimulated by NADH (2.2-fold; lane 2) and somewhat further in the presence of unlabelled reticulocyte lysate (40% over NADH alone; lane 4) or cytosol fraction (26% over NADH alone; lane 6); this stimulation over NADH-independent m-C₁ formation was inhibited by deuterohemin (lanes 3 and 5); formation of m-C₁ in the presence of Na dithionite (lane 7) was 85% of the amount observed with NADH (lane 2).

It therefore appears that the second processing step of cytochrome c₁ is affected by conditions which affect heme attachment to cytochrome c and thus, by analogy, might affect heme attachment to cytochrome c₁. It is possible then that heme attachment to cytochrome c₁ enhances the rate of the second processing step, though it might not be obligatory.

DISCUSSION

Cytochromes c and c₁ are imported into mitochondria by distinctly different mechanisms. They do, however, share the common requirement for covalent heme attachment during the final stages of their respective import pathways. During the import of cytochrome c, this step occurs in the mitochondrial intermembrane space (8) and is responsible for the completion of translocation of the protein across the outer membrane, probably owing to a conformational change resulting from the covalently attached heme group. To account for previous observations and those presented here we propose the following model. Apocytochrome c spontaneously inserts part way into the lipid bilayer of the outer membrane with low affinity (34-39). The partially inserted protein is then bound with high affinity by a binding protein which is localized in the intermembrane space (Köhler, Stuart and Neupert, in preparation). Heme is covalently attached to the cysteine thiols at positions 14 and 17 (universal numbering) in a reaction which is catalyzed by the enzyme cytochrome c heme lyase and is dependent on NADH plus a cytosolic cofactor. The NADH appears to be required for reduction of the heme iron to Fe(II) before this step, though it is unclear where and how this might occur (Nicholson and Neupert, in preparation). The covalently attached heme group is presumably then the nucleus around which the rest of the polypeptide chain folds, drawing the remainder of the molecule across the outer membrane as it does so to its final location in the intermembrane space.

Since cytochrome c₁ maturation shares so many common features with the heme-attaching step in cytochrome c import, it might be expected that the same enzyme catalyzes the reaction for both. Cytochrome c₁, like cytochrome c, is also exposed to the intermembrane space where heme attachment occurs in the latter case. Furthermore, there is sequence homology between the two proteins in the heme-binding region, e.g. in *N. crassa* (40-42):

Cytochrome c (n)...KTRCAQCHITLEEG...(c)
 Cytochrome c₁ (n)...REVCA~~Q~~~~C~~~~H~~~~I~~~~T~~~~L~~~~E~~~~E~~~~G~~SRV...(c)

Despite these likely circumstances, however, a yeast mutant which lacks the heme lyase enzyme for cytochrome c (cyc3⁻) contains normal levels of cytochrome c₁ (43,44). We have found this to also be the case for a mutant of *N. crassa* (cyt-2-1) that is deficient in cytochrome c heme lyase activity, and therefore contains no cytochrome c, but which has normal levels of cytochrome c₁ (Nargang, Nicholson and Neupert, in preparation). It therefore appears that two separate enzymes are involved, perhaps because of topological requirements during import and maturation.

How might the covalent attachment of heme to cytochrome c₁ be related to the second proteolytic processing event? It is apparent that heme attachment must precede processing of i-C₁ to m-C₁. This is indicated by the requirement of this step for NADH and its inhibition by deuterohemin. Similarly, a heme-deficient mutant of yeast accumulates i-C₁ in vivo (25-26). One possibility is that heme attachment affects the conformation of i-C₁ around the region of the second processing site, which enables recognition by the second processing protease. A more likely possibility is that following heme attachment and the ensuing conformational changes, the second processing site becomes accessible to the peptidase. For example, applying the principles of the 'stop transport' model (45), the following sequence of events might occur. The precursor of cytochrome c₁ binds to its receptor at the outer mitochondrial membrane and the N-terminus is translocated through the inner membrane, via contact sites, in a step which is dependent on a membrane potential. The positively charged first part of the prepiece is removed by the chelator-sensitive matrix peptidase leaving the more hydrophobic 'stop transport' domain (the second part of the prepiece) inserted through the inner membrane. At some point following translocation of the remainder of the protein across the outer membrane, the C-terminus (which anchors the mature protein to the inner membrane) becomes embedded in the inner membrane. At this stage the second processing site is presumably not accessible to the intermembrane-space-localized second processing protease. Following covalent heme attachment, however, the resulting conformational change might expose this site allowing the removal of the remainder of the prepiece to occur.

Another possibility is that cytochrome c₁ follows a 'conservative sorting' pathway similar to that of the Fe/S protein of the bc₁ complex (46) in which p-C₁ would be transported completely into the matrix where the first part of the prepiece is removed. The second part of the prepiece might then redirect i-C₁ back to the inner membrane. Covalent heme attachment and the ensuing conformational change is then responsible for drawing the protein back across the inner membrane, exposing the second processing site in the meantime. As the C-terminus is pulled through it anchors the protein to the inner membrane. If this pathway were to be the case, then the coupling of translocation to heme attachment for cytochrome c₁ might be analogous to the same event during cytochrome c import, except that instead of being translocated across the outer membrane into the intermembrane space (as is the case for cytochrome c), cytochrome c₁ is translocated from the matrix across the inner membrane to the intermembrane space. The intermembrane space i-C₁ should therefore have heme attached to it while the matrix localized i-C₁ species should not. Further work is required in this regard to determine the exact route that cytochrome c₁ takes during import. Where and how the heme attaching step occurs, and how it compares with the equivalent step of cytochrome c import will also be of considerable interest.

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